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Anion pores from magainins and related defensive peptides

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Abstract

The conformations and properties of magainins, powerful antimicrobial peptides isolated from *Xenopus laevis* skin, are reviewed. Although some emphasis will be placed upon membrane interactions both in living cells and liposomes, we will especially focus on pore-forming properties as studied with conductance measurements on doped planar lipid bilayers. The relevance to channel modelling and engineering will be stressed and finally the relations, analogies and differences, of magainins with other insect and mammalian defensive peptides, such as *cecropins* and *defensins*, will be presented.

Key words: Magainins; *Cecropins*; *Defensins*; Membrane permeabilization; Ion channels; Liposomes; Planar lipid bilayers

1. Introduction

In the course of evolution, living organisms have devised fast and general purpose mechanisms of defense. As the ubiquitous heat shock or stress proteins are designed to quickly cope with physical or chemical aggressions (Schlesinger et al., 1982), before the system can readapt on a longer term through metabolic pathways and/or readjustment of membrane composition and viscosity (the 'homeoviscous adaptation' (Cossins and Sinensky, 1984), a fixed register of various peptides can be mobilized to fight with a large spectrum microbial invasions.

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1

In invertebrates characterized by a high rate of reproduction and a short life span and where the primitive immune system is apparently not able to synthesize immunoglobulins, these peptides constitute the main defensive system. They have been remarkably conserved throughout evolution since some *cecropins* and *defensins*, for example, originally described in insects, have also been characterized in mammals. However, the latter are usually shorter and their structure may be different, such that a common ancestral gene can be ruled out.

There was literally a boom, in the last decade, in the number of articles dealing with defensive peptides, the 'Magainin family' corresponding to medium-sized peptides whose active structure is mainly α-helical. The circumstances of the discovery of antimicrobial peptides in *Xenopus* are recalled in the first part of this review. From the structures, in solution and in membranes, of magainins and analogues, it will be apparent that their large antibacterial spectrum is correlated with α-helical content. The 'membrane properties' of magainins will then be summarized, proceeding from the general or integrated ones on living cells (loss of respiratory control, cytolysis...) and model systems (liposomes destabilization) to the molecular level, stressing ion channel formation. Finally, structural and functional analogies of magainins with insect and mammalian related defensive peptides will be outlined. An earlier and excellent review was already available (Berkowitz et al., 1990).

2. The search for antimicrobial factors in the amphibian skin

The oocytes from the south African clawed frog have long been used in developmental biology (Dumont et al., 1972) and more recently and widely for injecting exogenous mRNA in order to follow the expression of various receptors and channels (Sigel, 1990). To the scientist involved in those studies, it is a common observation that, despite the non-sterile surgical removal of oocytes through an incision in the skin and the abdominal muscular layers, followed by sutures and return to holding tanks, the wounds heal within a few weeks without any special care. Although these amphibian are endowed with a competent immune system (Evans, 1963), there are no signs of inflammation or cellular reactions.

Zasloff (1987) suggested that there might be some unknown factors in the amphibian skin underlying this remarkable 'sterilizing' activity and this induced him to undertake a systematic search. In a seminal paper (Zasloff, 1987), using skin extracts, peritoneal and subdermal fluids (but not from skin superficial secretions as initially thought), the authors detected an inhibition of bacterial growth on an agarose support. The *Escherichia coli* strain which

was used in those tests was a 'liposaccharide mutant', considered more sensitive to membrane-active antibiotics.

Ion exchange chromatography followed by gel filtration and HPLC allowed the isolation of specific active peptidic fractions whose molecular weight, as revealed by electrophoretic patterns, was between 2 000 and 3 000 Da. The two most active components, designated as Magainins (from the hebrew 'Magain' meaning 'shield', and abbreviated thereafter as Mag) are 23-residue peptides of nearly identical sequences (see Table 1). The net charge, due to four lysines and a free *C*-terminus is the same for both peptides, but while those *Lys* are more or less evenly distributed along Mag 1 sequence, they are somewhat clustered (three in the middle) in Mag 2, leaving a neutral *C*-terminal. Finally, the sequence of a partial cDNA for precursors argues that both peptides derive from a common larger polypeptide (Zasloff, 1987).

3. Other antimicrobial peptides secreted by granular glands from *Xenopus*

Magainins are only members of a diverse population of antimicrobial peptides within the amphibian neurosecretory system since, although not sharing an extensive sequence homology with magainins but of roughly similar size, amphipathy, richness in basic residues (4–5 *Lys*) and extracted from the same tissues, at least two other peptides were later found to exhibit a broad spectrum of antibacterial activity (Soravia et al., 1988). These are termed PGLa and XPF (Table 1), 21 and 25 residue peptides, respectively, deriving from the *Xenopsin* precursor, also found more recently in the gastrointestinal tract (Sadler et al., 1992).

Using the same assays developed for magainins (inhibition of an *E. coli* lawn, O.D. of several bacteria and fungi species in liquid medium, osmotic lysis of protozoan species), all four peptides showed similar potency, the minimal inhibitory concentration being in the 5–50 μg/ml range. However, some differences were noted, in particular Mag 2 and XPF displayed a higher specific activity against *Pseudomonas* than did PGLa which was mostly efficient against *Staphylococcus aureus*. This seems somewhat unexpected, since the sequences homology is much higher between XPF and PGLa than with magainins. By contrast, the hemolytic activity against human erythrocytes is very low (Soravia et al., 1988).

4. Conformations of magainins

Upon inspection of the primary structures of magainins, it was soon postulated that they might form amphipathic α-helices of length sufficient enough to cross a membrane bilayer.

Table 1
Amino-acids sequence of magainins and some related antimicrobial peptides

Magainin family	
Magainin 1:	GIGKFLHSAGKFGKAFVGEIMKS
Magainin 2:	GIGKFLHSAKKFGKAFVGEIMNS
XPF:	GWASKIGQTLGKIAKVGLKELIQPK
PGLa:	GMASKAGAIAGKIAKVALKAL(NH_2)
Cecropins	
Insects	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK(NH_2)
(Hyalophora):	
Pig	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR
intestine	
Defensins	
Insects	ATCPLLSGTGINHSACA-AHCLLRGNRGGYCNGKGVCVCRN
(Phormia)	
Mammals	VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR
(Rabbit NP-1)	

Sequences have been aligned for optimal homologies.

A 2D proton-NMR study confirmed that whilst the conformation of magainin 2 in water was random coil and temperature-insensitive, the addition of small amounts of trifluoroethanol (TFE), through a reduced solvent-polarity, stabilized intramolecular hydrogen bonding involving the entire chain (Marion et al., 1988). This solvent, mimicking a membrane environment, was justified by the similar conformations obtained in water-TFE mixtures and dodecylphosphocholine dispersions.

Although an α-helical structure with a large hydrophilic sector, comparable to melittin (Habermann and Jentsch, 1967) is a priori compatible with the building-up of transmembrane aggregates managing an ion pore within them (Fox and Richards, 1982), circular dichroism spectroscopy of magainin 1 in lipid vesicles (Duclohier et al., 1989) subsequently revealed significant differences with alamethicin, the prototype of such channels.

Whereas the helical content of alamethicin in phosphate buffer is significantly increased (30%–35%) upon addition of egg lecithin vesicles, the magainin-induced helicity is much more modest (10%), even more when negatively-charged lipids are incorporated into the vesicles (egg PC/POPS: 7/3). Although there seems to be some discrepancy about the latter point (Matsuzaki et al., 1989), this suggests a strong electrostatic interaction of Lys residues with negatively-charged headgroups, magainins lying flat on top of

the bilayer, in an extended conformation, especially for carboxy-amidated analogues (Jackson et al., 1992). *H*-bonding could then be satisfied between neighbouring peptides. The latter study using Fourier transform infrared spectroscopy also points out some β-sheet contribution (presumably reflecting the portion of the peptide ouside the bilayer) favored by cholesterol. This is confirmed in a recent solid state ^{15}N-NMR investigation also stressing the parallel orientation of magainin to the bilayer surface (Bechinger et al., 1992).

5. α-Helical content associated with antimicrobial and hemolytic activity

A systematic study aimed at enhancing the magainin helical content by substituting low helical-propensity residues along the sequence, Ser_8, Gly_{13} and Gly_{18} with Ala (Chen et al., 1988). Furthermore, the *C*- and *N*-termini were, respectively, amidated and acylated to stabilise the helical conformation. Circular dichroism measurements in aqueous phosphate buffer and 40% trifluoroethanol confirmed that the helical content of the analogue was doubled as compared to magainins 1 and 2 (24%–26%), while the acetylation of Gly_1 alone was without effect. This was associated with a parallel increase of up to 2 orders of magnitude in antimicrobial activity with a conserved spectrum. Ser_8 appeared a less 'sensitive' residue than Gly_{13} and Gly_{18} since its non-substitution by Ala only slightly reduced antimicrobial activity. In addition, these studies suggested that a free *N*-terminus (i.e. non-acetylated) was required to elicit the maximal antimicrobial activity (Chen et al., 1988).

These analogues had also an increased hemolytic activity over the natural magainins (which are virtually inactive). However, an appreciable hemolysis was only obtained at 100 μg/ml, i.e. equivalent to 1% the potency of melittin. Here, Ser_8 seems the most important residue (putative phosphorylation site).

Finally, in connection with the eventual values of magainins in antibiotic therapy, it should be mentioned there is a remarkable synergy between these peptides and β-lactam antibiotics. Whilst Mag 2 alone was inefficient in the treatment of mice infected with *E. coli*, the survival was significantly increased when used in conjunction with cefepime for example (Darveau et al., 1991).

6. Cytolysis, depolarization and other biological effects

In addition to their antibiotic activity (through depolarization) against various microorganisms, magainins have been more recently shown to irreversibly lyse various hematopoietic tumor and solid cells at concentrations

which are relatively nontoxic to well-differentiated cells (Cruciani et al., 1991). The relative antitumoral activities of the different tested analogues were similar to their antibiotic potencies, although in terms of absolute concentration, the more potent peptides were 5–10 times less effective against the tumor cells than against *Escherichia coli*.

In the same study, membrane potential shifts in tumor cells stained with the fluorescent potentiometric oxonol dye (calibrated with the cation-selective ionophore gramicidin and various external sodium concentrations) are consistent with the notion that cytotoxic concentrations of magainins induce poorly selective ion channels. In agreement with conductance experiments (see below), a negative membrane potential seems critical for channel formation since depolarization prevents the cytotoxic activity of magainins (Cruciani et al., 1991). However, the resistance of well-differentiated cells such as peripheral blood lymphocytes and polymorphonuclear neutrophils to lysis remains to be explained.

Differences in membrane composition, membrane potential or surface charges as well as proteolytic defense of the (eukaryotic) target cells have been put forward without much experimental evidence. Another hypothesis suggested that magainins might interfere with membrane-linked free-energy transduction (de Waal et al., 1991) and made use of spermatids and spermatozoa (from hamsters) as a test system. Indeed, these cells have to rely on lactate oxidation, and thus on mitochondrial metabolism, for ATP production. Release of respiratory control and inhibition of oxygen uptake in the presence of excess uncoupler, indicative of inhibition of electron transfer through the respiratory chain, correlated with the loss of spermatozoa motility and with membrane depolarization. Furthermore, these effects were half-maximal at about 4 μg/ml magainin, equivalent to an average antibacterial concentration (de Waal et al., 1991).

7. Interaction with liposomes: Membrane permeabilization

Liposomes are useful for elucidating peptide-lipid molecular interactions since signal/noise ratios associated with various spectroscopic and thermal techniques applied to a large population of lipid vesicles allow correlations to be drawn with changes in membrane permeability, for example.

A first set of studies stressed the specific interaction of Mag 1 with acidic lipid vesicles (Matsuzaki et al., 1989). The same technique — the vesicular release of entrapped calcein upon interaction with the effector — supplemented with circular dichroism and differential scanning calorimetry was used in a subsequent study (Matsuzaki et al., 1991) extended to phosphatidylglycerols, abundant acidic lipids in bacterial membranes, and to Mag 2. The binding affinities of both peptides coincided with the zeta potential

order of the lipid vesicles and were inversely related to ionic strength. While electrostatic interactions thus played a predominant role in the initial binding process, the time course of the fluorescent probe release from the vesicles argued for a higher 'membrane perturbing activity' in the more fluid membranes (dioleoylphosphatidylglycerol and phosphatidylserine). Apparently, gel-state membranes (like dipalmitoylphosphatidylglycerol) with strong hydrophobic interactions inhibit a deep insertion of magainins.

While the negatively-charged *C*-terminal should be expelled from the membrane surface by electrostatic repulsion, the circular dichroism data suggested that 14–19 residues from the *N*-terminal domain could form amphipathic helices. The Mag 2 higher activity was ascribed to an additional lysine (position 10) in this domain.

As for the vesicle permeabilization mechanism, a peptide-induced disruption of the lipid structure was favoured over channels made up of aggregates. Accumulation of monomers would lead to site-site (lipid defects induced by peptide) interactions and 'leaky patches'. As stated, applied transmembrane potential differences might alter peptide conformations, orientations and aggregations in membranes (Matsuzaki et al., 1991). Another more recent study focusing on the early time course of carboxyfluorescein release also favoured the idea of a 'transiently destabilized bilayer' (Grant et al., 1992). However, it should be noted that the peptide concentrations used in those studies are much higher (by several orders of magnitude!) than in conductance measurements on planar lipid bilayers.

Indeed, from an analysis of binding isotherms of Mag 2 with PS vesicles, the 'critical number' of bound peptide monomers necessary to induce leakage is estimated at 0.06–0.14 per molecule of lipid (Grant et al., 1992). In other words, there is a maximum of 7–14 lipid molecules associated with one peptide monomer during the initial transient membrane destabilization. On the other hand, from the maximum macroscopic conductance values found in planar neutral lipid bilayers (Duclohier et al., 1989), the figure is 10^6 lipid molecules per monomer involved in transmembrane conducting aggregates.

8. Ion channel formation

Apart from these general biological effects and underlying them (at least partly), the demonstration of ionophore activities induced by magainins in artificial membranes added a further interest in these peptides since it allows comparative studies with other natural and synthetic channel forming peptides (CFPs, Sansom (1991)) or pore-forming cytolysins (Menestrina, 1991). Indeed, a number of antibiotic or cytolytic peptides (alamethicin, melittin, δ-lysin. . .) share a minimum length of about 20 residues and α-helical struc-

ture in membranes. Ultimately, inferences could be drawn about models for channel formation, gating, ion selectivity when correlating a particular chemical sequence and key-residues substitutions to specific functional modulations.

A preliminary study with Mag 2 reported channel formation in PS: PC bilayers at the tip of patch pipettes (Cruciani et al., 1988). The channels exhibited a wide range of unit conductance levels and a poor selectivity for anions. A more extensive investigation, both at the macroscopic and single-channel levels, but only with Mag 1, confirmed these trends (Duclohier et al., 1989). Macroscopic current-voltage (I-V) curves, recorded from PE:PC Montal-Mueller bilayers of large area typically incorporating a hundred channels, were asymmetric, i.e. a significant voltage-dependent conductance was only developed for negative potentials, the peptide being added to the *cis*- (or positive-) side of the membrane. This result had to be expected, taking into account the positive charge near the *C*-terminal and a previous study with charged alamethicin analogues (Vodyanoy et al., 1983; Hall et al., 1984). From the concentration-dependence (in the 10^{-6}–10^{-7} M range) of these macroscopic conductances and their voltage-dependence (which is rather moderate as compared to alamethicin, for example), the apparent mean number of monomers forming the channels was estimated, $N = 3$–6.

The hypothesis of an anionic selectivity, as expected from the high content of positively-charged residues (K) in the hydrophilic sector which are lining the pore lumen, was confirmed in experiments where a salt gradient was imposed to the bilayer doped with Mag 1. The resulting shift in the reversal (or zero-current) potential and the application of the Hodgkin–Goldman–Katz equation (Golman, 1943; Hodgkin and Katz, 1949) allowed the inference of a weak anionic selectivity with P_{Cl}/P_K estimated at 3.

With bilayers of the same composition (POPC:DOPE, 7:3) at the tip of patch-clamp pipettes, a statistical distribution of single-channel conductance levels was observed, i.e. within a single experiment, only one level was generally recorded but that level could differ from one experiment to another. The amplitudes of the two most probable levels (others being relatively rare and short-lived) averaged 680 and 360 pS (Duclohier et al., 1989). Lower and faster events seemed to be favoured in bilayers incorporating negatively-charged lipids. Note that this was matched by a reduced ellipticity (see above: 4. Conformations of magainins), the peptide likely to lie flat on the membrane surface.

It is also interesting to compare this behavior with the one displayed by physiological channels. For example, the anionic/cationic selectivity ratio for Mag 1 falls into the range of those reported for natural anionic channels in vertebrate twitch muscle (Hille, 1984). Many of them seem to function as twin gated units, for example in cultured cardiac cells, two large conductance

systems of comparable amplitude had been observed with a ratio of roughly 2 (Coulombe et al., 1987).

9. Related insect and mammalian host defense peptides: cecropins and defensins

Following the injection of bacteria into the hemolymph of the pupa, the humoral immune system of the North American silk moth *Hyalophora cecropia* and numerous other insects produces a class of antibacterial peptides known as *cecropins* (Boman and Haltmark, 1987).

Cecropins, despite being significantly longer (35–37 residues, although the more recently characterized analogue, in the pig intestine (Lee et al., 1989) is only 31-residue long) are quite related to magainins (see Table for sequences) since they share a broad antibacterial spectrum including Gram (+) and Gram (−) species through a lytic action, liposome permeabilization (Steiner et al., 1988) and finally anion-pore formation (Christensen et al., 1988).

At the structural level, they are also basic peptides, rich in lysines but, at least in insect *cecropins*, the helical pattern is interrupted by a central hinge (residues 12–24) separating a quite amphipathic α-helical *N*-terminal (with a large hydrophilic sector) and a rather hydrophobic *C*-terminal helix. This peculiar arrangement was postulated, in a study of synthetic cecropin-like model peptides (Fink et al., 1989), to be a prerequisite for antibacterial activity. Since the porcine cecropin shows a continuous amphipathic α-helix over most of its length, such a hinged structure might not be essential for activity although a bend could be initiated at the region Glu_{20}-Gly_{21}, especially on a membrane surface (Sipos et al., 1992).

Such a hinge was also invoked to account for the voltage-dependence of channels formed by *cecropins* (Christensen et al., 1988). Although it was argued that analogues with a more rigid chain did not exhibit any voltage-dependence, it is worth noting that the latter property with the most efficient cecropin (AD) remained quite moderate (Christensen et al., 1988) as compared to magainin 1 (Duclohier et al., 1989) which does not exhibit such helix breaking Gly-Pro motifs. Also in this connection, alamethicin synthetic analogues where Pro_{14} had been substituted by Ala conserved an impaired voltage-dependence (Duclohier et al., 1992). As for magainins, however, a negative voltage was required to induce cecropin channels which were anion-selective, with a permeability ratio $P_{Cl^-}/P_{Na^+} = 2$.

The incorporation of positively-charged lipids or cholesterol in planar lipid bilayers significantly reduced the observed conductances (Christensen et al., 1988). Note that both chemicals were required to confer liposomes resistance to lysis, cholesterol alone being ineffective (Steiner et al., 1988).

Extracted from insect hemolymph and from macrophages, granulocytes

and human neutrophils, the *defensin* peptides are also cationic and about 35–45-residue long (Table 1) with a number of disulphide bonds (Elsbach and Weiss, 1988). Insect and mammal *defensins* structures (Hill et al., 1991; Bonmatin et al., 1992) appear to be significantly different and thus the hypothesis of a common ancestral gene has to be excluded. The peculiar arrangement in insect *defensins* — a 10-residue long amphiphatic α-helix is linked to a 13-residue long β-sheet through 3 disulfide bonds — is equally found in toxins isolated from scorpion venoms (Bontems et al., 1991) to which they appear more structurally-related than to their mammalian counterparts (Hoffmann and Hetru, 1992).

By analogy to the above mentioned toxins, there appears to be various modes of action. *Defensins* are believed to be active mainly intracellularly being inactivated by extracellular serum: a 1-minute contact with 0.5 μM insect defensin is sufficient to kill Gram-positive germs (Lambert et al.,1989). In contrast to magainins and *cecropins*, few Gram-negative cells are affected by these peptides. The exact mode of action has not yet been fully elucidated, but the high affinity with cardiolipin seems to be essential (Matsuyama and Natori, 1990).

Indeed, membranes are once more likely to be the primary target since channel formation has been reported for NP-1, the rabbit defensin (Kagan et al., 1990), in a concentration range (0.1–50 μg/ml) comparable to the one required for in vitro antimicrobial activity. These channels were turned-on only by negative voltage, but the convention sign was the opposite of the usual one, such that, when compared to magainins, the defensin channels appear to be activated by opposite voltages. On the other hand, the voltage-dependence was quite similar to the magainin one, the conductance increasing every 23 mV. Likewise, they were also found to favour Cl^- over Na^+ and K^+ with a permeability ratio equivalent to those displayed by *cecropins* and magainins. At the single-channel level, the rule was the heterogeneity of unitary conductances ranging from 10 to 1 000 pS. This behaviour and the concentration- and voltage-dependences of defensin-induced conductances argued for multimeric (N = 2–4) channels (Kagan et al., 1990).

10. Conclusion

In addition to representing a promising avenue for therapeutic application, magainins, *cecropins* and *defensins* have already provided interesting outcomes as far as ion channels mechanisms are concerned. Magainins and *cecropins* were the first peptides reported to form anion-selective pores through monomers aggregation. The demonstration of channel activity with *defensins* confirms that non-helical structures are to be considered in the investigation of structure-function relationships of not only porins (Pauptit et

al., 1991) of course, but also of other physiologically important ionic channels (Sansom, 1993). In particular, α/β motifs (association of α-helices and β-sheets) are recently assumed to form the central core of the acetylcholine receptor (Unwin, 1993) as well as of voltage-gated channels (Stephan and Agnew, 1991; Pongs, 1992).

Cecropin channels were recently subjected to an atomic-scale modelling (Durell et al., 1992) which depicts the transition between two open conformations, as experimentally observed (0.4 and 1.9 nS in 0.1 M NaCl, (Christensen et al., 1988): the type I channel with the pore formed by transmembrane *C*-terminal helices leads to larger channels formed by *N*-terminal helices (type II channel). A hexagonal lattice of type I channel is proposed (Durell et al., 1992) to explain the large number of peptides binding the bacterium and inducing its lysis. This should not be exclusive of lipid defects resulting from the high surface density of helices.

Potent antimicrobial (both against Gram-positive and Gram-negative bacteria) amphipathic peptides were designed and synthesized according to the magainin skeleton. The only residues were leucine and lysine (in doublets). Retention times in reverse phase HPLC were found to correlate with antibiotic activity and hemolysis was favoured by greater hydrophobic interactions, i.e. when leucines replaced some charged residues (Blondelle and Houghten, 1992).

Finally, plants are not exempted from such bioactive compounds since two related 29-residue antimicrobial peptides have been recently isolated from seeds of amaranth (Broekaert et al., 1992).

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becco's minimum essential medium (DMEM) in the presence of polybrene (8 μg/ml) as described (*18*). Cells were washed twice in phosphate-buffered saline (PBS), resuspended in fresh medium, and cultured for 3 to 4 days. Transduced cells were tested for the presence of helper virus and cryopreserved until use.

36. BM mononuclear cells were obtained as a Ficoll fraction and grown for 2 to 3 days in complete DMEM at a density of 6×10^5 to 8×10^5 cells/cm^2 (*35*). T cell depletion and progenitor cell enrichment were obtained as described (*3, 35*). Gene transfer was carried out by multiple infection cycles with cell-free, helper virus–tested viral supernatants in the presence of polybrene (8 μg/ml) (*35*). BM cells were maintained in a long-term culture system over adherent layers without addition of exogenous growth factors, and infected during the first 3 days of culture. Transduced cells were tested for the presence of helper virus and cryopreserved until use. At that time, the transduced cells were washed, resuspended in normal saline containing 4% human albumin, and reinfused into the patient.
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38. PHA blasts or antigen-specific T cells were cloned by limiting dilution. The relative frequencies of transduced cells was obtained by comparing the precursor frequency in the absence and presence of G418 (800 μg/ml). G418-resistant T cell clones were isolated and maintained as described (*44, 45*).
39. The relative frequencies of transduced BM progenitor cells were obtained by comparing the frequency of CFU-G, CFU-GM, BFU-E, and CFU-GEMM cells in the absence and presence of increasing doses of G418 (0.7, 1.0, 1.5 mg/ml) as described (*37*). In selected experiments, individual G418-resistant colonies were collected for analysis of vector transduction and expression.
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48. T cell receptor V_β-chain usage was analyzed on transduced T cell lines by reverse transcriptase–PCR. Briefly, total RNA was reverse transcribed with oligo(dT) and oligo(dG) primers and subjected to PCR with V_β- or C_μ-specific oligonucleotides (*46*) or to anchored PCR with a C_μ-specific oligonucleotide as described (*47*). Amplified products were analyzed by agarose gel electrophoresis.
49. We are indebted to L. Ruggieri and A. Wack for performing some of the ex vivo and in vitro analyses of gene transfer frequency; to the nurses and clinical staff of the Clinica Pediatrica, School of Medicine, University of Brescia, for skilled and dedicated care; to A. Arrighini and A. Crescenzo for clinical assistance in the extended care of the two patients; to A. Plebani for dosing specific antibody production; to M. Hershfield, P. Dellabona, and A. Ballabio for helpful discussions; and to Enzon, Inc., and Ophan Europe for providing PEG-ADA before commercial distribution. Supported by grants from Telethon, the Italian National Research Council, and the Italian Ministry of Health (IV-VII AIDS Projects).

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T Lymphocyte–Directed Gene Therapy for ADA$^-$ SCID: Initial Trial Results After 4 Years

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In 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA$^-$ SCID). The number of blood T cells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.

The possibility of using gene transfer as a therapy for human disease has great appeal. The decision to enter clinical trials awaited the development of safe and efficient techniques of gene transfer and improved understanding of the basic pathology and biology underlying likely candidate diseases and target cells. The advent of useful retroviral vectors that permitted relatively high efficiency gene transfer and stable integration was a critical advance (*1, 2*), as was the demonstration that this procedure of gene transfer could be effectively and safely used in humans (*3*).

Severe combined immunodeficiency secondary to a genetic defect in the purine catabolic enzyme adenosine deaminase [ADA$^-$ SCID] is characterized by defective T and B cell function and recurrent infections, often involving opportunistic pathogens. Large amounts of deoxyadenosine, an ADA substrate, are present in these patients; deoxyadenosine is preferentially converted to the toxic compound deoxyadenosine triphosphate in T cells, disabling the immune system (*4*).

Because this disease is curable by allogeneic bone marrow transplantation given without pretransplantation cytoreductive conditioning, it was initially assumed that gene therapy should be directed at the bone marrow stem cell. However, initial attempts to use stem cell gene transfer in primates resulted in only low-level, transient gene expression, insufficient for clinical use. The observation that the only donor cells detected in some patients "cured" by allogeneic bone marrow transplantation was their T cells—the others remaining ADA-deficient (*5*)—raised the possibility that T cell–directed gene therapy also might be a useful treatment.

The introduction of enzyme replacement with ADA-containing erythrocytes (*6*) or with bovine ADA conjugated with polyethylene glycol (PEG-ADA) (*7*) has made this approach feasible. PEG-ADA has provided noncurative, life-saving treatment for ADA$^-$ SCID patients; with this treatment, most patients have experienced weight gain and decreased opportunistic infections. Full immune reconstitution has been less regularly achieved with enzyme therapy. T cell function as measured by in vitro mitogen responses improved in most patients, but fewer patients recovered consistent immune responses to specific antigens [for instance, as measured by normal delayed-type hypersensitivity (DTH) skin test reactivity] (*8–10*). Nearly all PEG-ADA–treated patients showed increased peripheral T cell counts, which provided a source of T cells for gene correction not available without enzyme therapy. Furthermore, enzyme treatment could be continued during the gene therapy trial so that the ethical dilemma of withholding or stopping a life-saving therapy to test an unknown treatment could be avoided.

The adenosine deaminase complementary DNA (cDNA) (*11*) is 1.5 kb and fits within a retroviral vector. With the use of an ADA-containing retroviral vector, ADA-deficient T cell lines were transduced to express normal amounts of ADA; this rendered them normally resistant to intoxication and growth inhibition when challenged with deoxyadenosine (*12, 13*). Next, studies in mice, rabbits, and nonhuman primates using T cells modified with retroviral vectors showed normal cell survival and function after their reintroduction into recipient animals (*14*). Finally, Bordignon and colleagues (*15*) showed that ADA gene–corrected T cells acquired a survival advantage compared with uncorrected ADA-deficient cells when transplanted into immunodeficient, but ADA-

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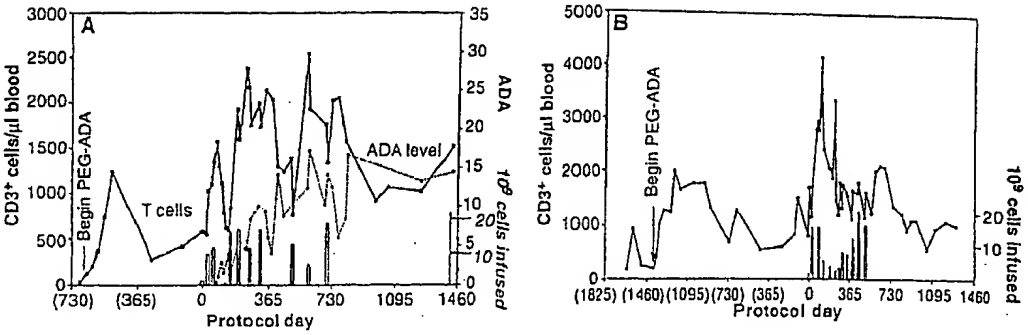

1. Peripheral blood T cell counts ce the time the diagnosis of ADA ficiency was made, dates of treatments, and the total number of cells infused for each patient. ADA level is measured in nanomoles of adenosine deaminated per minute per 10^8 cells. Vertical bars indicate the dates of cell infusion, and their height represents the total number of nonselected cells infused at each treatment. The T cell numbers represent total CD3-bearing T cells determined by standard flow cytometric analysis. (A) Patient 1 began gene therapy on 14 September 1990 (protocol day 0) and received a total of 11 infusions. Cellular ADA enzyme level is indicated by the dashed line. ADA activity was determined as described (*13*, *25*). Values shown are the mean of duplicate samples and represent EHNA-sensitive ADA enzyme activity. (B) Patient 2 began gene therapy on 31 January 1991 (protocol day 0) and received of a total of 12 infusions.

normal BNX recipient mice.

The clinical protocol used here has been described elsewhere (*16*). Patients with documented ADA$^-$ SCID were eligible if they did not have a human lymphocyte antigen–matched sibling as a potential donor for marrow transplantation and if they had been treated with PEG-ADA for at least 9 months without full immune reconstitution. T cells were obtained from their blood by apheresis, induced to proliferate in culture, transduced with the ADA retroviral vector LASN, culture-expanded, and then reinfused into the patient after 9 to 12 days (*17*). No selection procedure was used to enrich for gene-transduced cells.

The clinical histories and ADA gene mutations of each patient have been reported (*18*, *19*). Patient 1 presented with infection at 2 days of age and had recurrent infections and very poor growth until 26 months of age, when the diagnosis of ADA deficiency was established and she was started on PEG-ADA [30 U per kilogram of body weight per week (30 U/kg/week)]. Treatment with PEG-ADA enzyme for approximately 2 years had resulted in significant, but incomplete, benefit. With PEG-ADA she gained weight, had fewer infections, and transiently developed a normal peripheral blood T cell count (Fig. 1A), and her T cells had acquired the ability to respond to mitogens in vitro. However, significant immune deficiency persisted, including recurrence of her T lymphopenia (Fig. 1A), DTH skin test anergy (Table 1), depressed in vitro immune reactivity to specific antigens such as tetanus toxoid, failure to generate normal cytotoxic T cells to viral antigens or allogeneic cells, defective immunoglobulin production and absent or weak antibody responses to several vaccine antigens, and borderline isohemagglutinin titers (Table 1). At 4 years of age, she was enrolled in this trial.

The course of disease in patient 2 (who was 9 years old when enrolled in the trial) was milder than that seen in classic SCID (*19*). She had her first serious infection at age 3, and septic arthritis at age 5; the diagnosis was finally established at age 6 when significant lymphopenia with ADA deficiency was confirmed. This patient had an excellent initial improvement in peripheral T cell numbers after the start of PEG-ADA therapy (30 U/kg/week) at age 5, but lymphopenia recurred in the third and fourth years of enzyme treatment (Fig. 1B). During the year before gene therapy, repeated evaluation of her immune system showed persisting immunodeficiency, but less severe than that in patient 1. Despite 4 years of enzyme treatment, DTH skin test reactivity was absent (Table 1), cytotoxic T cells to viral antigens and allogeneic cells were deficient, and isohemagglutinins were barely detectable. However, illustrating the variability seen in the responses of patient 2 over time, blood lymphocytes that were cryopreserved from the day the clinical trial began and tested later showed normal cytotoxic activity to allogeneic cells.

Within 5 to 6 months of beginning gene therapy, the peripheral blood T cell counts for patient 1 (Fig. 1A) rapidly increased in number and stabilized in the normal range and have remained normal since that time (*20*). ADA enzyme activity, nearly undetectable in her blood lymphocytes initially, progressively increased in concentration during the first 2 years of treatment to reach a level roughly half the concentration found in heterozygous carriers (expressing only one intact ADA allele) and has re-

Table 1. DTH skin test reactivity and isohemagglutinin titers in sera of each patient at various times during the treatment protocol. Skin tests were applied as Multitest (Pasteur Merieux, Lyon, France) and scored according to the manufacturer's instructions 48 to 72 hours after being placed. Seven antigens were placed on the dates indicated, although only five were technically satisfactory on day 1252 for patient 1 and on day 1118 for patient 2. Isohemagglutinin titers were determined by standard blood bank techniques (*34*). Ninety five percent of normal children over the age of 2 years will have a titer of ≥1:16 and 82% will have a titer ≥1:32 (*35*). ND, not done. For the DTH skin tests, positive tests were ellcited; T, tetanus toxoid; D, diphtheria toxoid; C, *Candida albicans*; P, Proteus antigen; S, streptococcal antigen; OT, old tuberculin.

Protocol day	Isohemagglutinins	DTH skin tests
	Patient 1	
−9	16	None (0/7)
115	256	ND
251	128	ND
314	32	T, D, C
455	32	T, D, C, S, P
510	64	ND
707	32	ND
1252	ND	D, C, P
	Patient 2	
−122	4	None (0/7)
−9	4	ND
90	256	ND
186	128	ND
291	128	ND
501	128	T, D, C, S, OT
676	64	ND
957	16	ND
1118	ND	T, D, S, P

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at that level since (Fig. 1A). Thus, the reconstituted number of peripheral T cells and the elevated T cell ADA enzyme concentration have persisted since patient's last treatment, indicating that peripheral T cells can have an unexpectedly long life-span and that gene expression from the retroviral vector has not been silenced over this period.

Patient 2, who had variable immune reactivity before enrollment, responded to the institution of lymphocyte infusions, with her peripheral T cell count rapidly increasing to levels in the high normal range (Fig 1B). Beginning with infusion 5, which included protocol modifications to partially deplete CD8 cells from the initially cultured cell population (*21*), her T cell count fell into the mid-normal range, where it persisted throughout the treatment period and for a year after the last cell infusion. In contrast to those in patient 1, ADA enzyme levels in the circulating T cells of patient 2 did not rise significantly above the small amounts seen before gene therapy treatment (~1.5 nmol/10^8 cells per minute).

The differences in final lymphocyte ADA concentration are consistent with the levels of gene transfer reached in these patients. For several months in the second protocol year during which cell infusions were not given, LASN vector sequences detected by polymerase chain reaction (PCR) maintained a stable frequency in the peripheral blood of patient 1 at a level greater than the PCR-positive control standard containing the equivalent of 0.3 vector copies/cell (Fig. 2). By contrast, although vector-containing cells were also stably detected throughout a similar period in patient 2, their level reached only a value equivalent to 0.1 to 1.0% of her circulating cells carrying the inserted ADA vector.

The principal contributor to the difference in the final frequency of LASN vector–modified T cells in patients 1 and 2 was the low gene transfer efficiency in the cells of patient 2; this was consistently only a tenth or less of what was routinely achieved in the cells from patient 1. Despite the gross differences in the final proportion of vector-containing cells reached in these two patients, both CD4 and CD8 T cell populations from each have remained consistently positive for integrated vector sequences since the first infusion through protocol day 1480 for patient 1 and through protocol day 1198 for patient 2 (Fig. 2).

To more accurately measure the proportion of vector-containing cells in patient 1, we performed quantitative Southern (DNA) hybridization analysis for vector sequence on DNA isolated from her peripheral blood T cells at different days during the course of this protocol. On protocol days 816 and 1252, which represent samples taken 109 and 545 days after the last treatment, the vector concentration was at the level of approximately one vector copy per cell (Fig. 3). Longitudinal studies of samples obtained throughout the study show that this large amount of integrated vector was reached by infusion 8 (D707) and that it has remained in this range since that time (*22*).

The use of a restriction endonuclease that cuts only once within the vector sequence does not give detectable bands (Fig. 3), indicating that the population of blood T cells at these dates is not oligoclonal with respect to integrated vector. Vector-derived mRNA was readily detected by reverse transcription (RT)–PCR at these same times (Fig. 3), confirming that vector expression persisted and was correlated with the presence of ADA enzyme activity in her circulating T cells.

To evaluate the effect of gene therapy on the immune function of these two patients in addition to its beneficial effect on T cell numbers, we performed a panel of immunologic studies both before, and at various times after, treatment. DTH skin test reactivity to common environmental and vaccine antigens tests the overall competence of the cellular immune system because a response depends on the full complement of cellular functions, not just cell proliferation or secretion of a single cytokine (Table 1). Patient 1 was anergic before our protocol treatment despite nearly 2 years of PEG-ADA treatment. Eight months after the initiation of gene therapy (protocol day 251), she had a brisk DTH response to a single intradermal skin test with tetanus toxoid. By protocol day 455, DTH responses to five of seven antigens were present, and this increased responsiveness has persisted, through day 1252.

Before the protocol, patient 2 had no positive DTH skin test (Table 1). At protocol day 501, five positive DTH skin tests were elicited, and this increased DTH reactivity had persisted when she was last tested on day 1118. She also acquired palpable lymph nodes and visible tonsils during the period of protocol treatment.

To corroborate the improved immune function indicated by these DTH tests, we evaluated the capacity of peripheral T cells from our patients to produce interleukin-2 (IL-2) or to kill antigenic target cells in vitro. In several patients treated with PEG-ADA, in vitro T cell proliferative responses to mitogens may normalize, whereas responses to specific antigens are less improved (*7–10*). During PEG-ADA treatment before gene therapy, T cells from patient 1 produced IL-2 in response to stimulation with

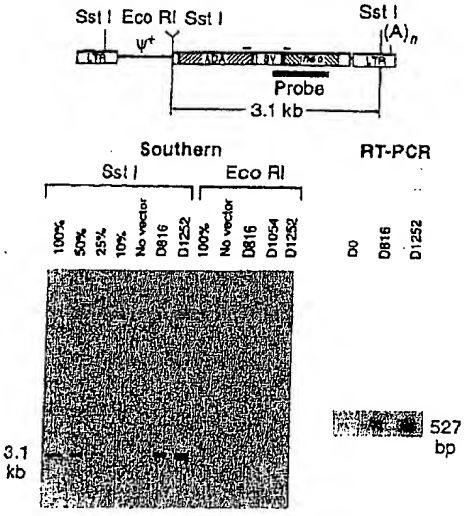


Fig. 3. Quantitative Southern hybridization analysis of DNA prepared from the blood mononuclear cells of patient 1 on protocol days (D) 816 and 1252 (*28*). DNA digested with Sst I should yield a single restriction fragment of 3.1 kb containing both the vector *neo* and ADA genes. Eco RI cuts only once within the vector sequence, and therefore a detectable band would indicate that a predominant clone with a single unique vector integration site was present in that blood sample. None was detected. Polyadenylated mRNA was extracted from the patient cells on days 0, 816, and 1252 and analyzed for vector message by RT-PCR (*29*). The primer locations used are indicated as short solid lines above the vector diagram. SV, SV40 early promoter; $(A)_n$, polyadenylation site; Ψ, extended retrovirus packaging signal. Hatched regions indicate protein coding regions.

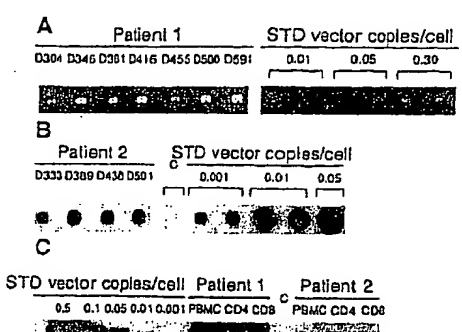


Fig. 2. PCR evaluation of the frequency of LASN vector–positive cells in the blood of patients 1 and 2 at various protocol days. (A) Cells from patient 1 for protocol days (D) 304 to 591 (see Fig. 1A). PCR analysis was performed as described (*26*) in an ethidium-stained gel. (B) Cells from patient 2 for protocol days (D) 333 to 501 (see Fig. 1B). PCR products were probed with ^{32}P-labeled *neo* gene as described (*26*). (C) Purified CD4$^+$ and CD8$^+$ cell subpopulations from patient 1 (D1480) and patient 2 (D1198) prepared by separation of peripheral blood mononuclear cells (PBMCs) by fluorescence-activated cell sorting (FACS). The purity of the separated T cell subpopulations from which DNA was extracted exceeded 98%, as confirmed by FACS analysis. Direct PCR with [^{32}P]deoxycytosine triphosphate was performed as described (*27*). Standards (STD) were prepared from DNA obtained from cell mixtures of a known proportion of LASN-transduced cells containing a single vector insert mixed with vector-negative cells. C, vector-negative control cells.

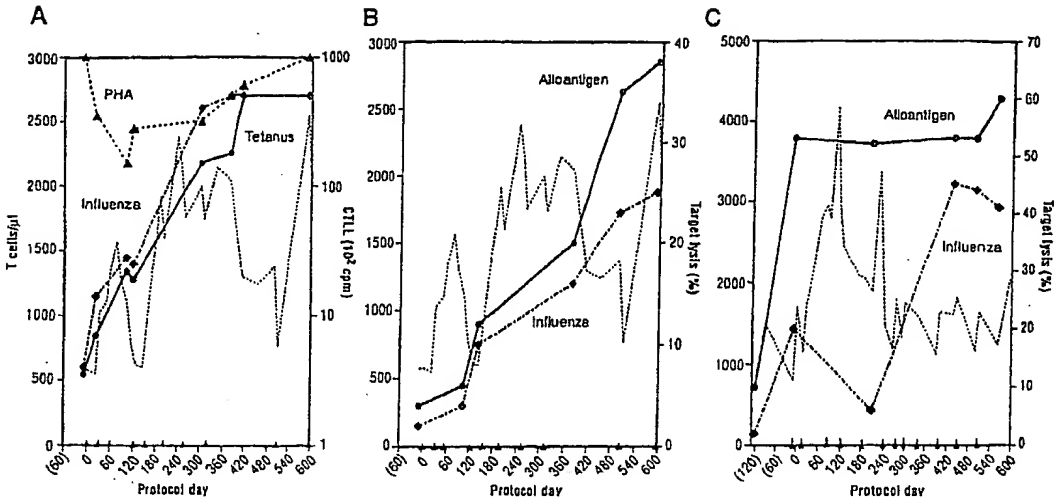


Fig. 4. Evaluation of the in vitro cellular immune responses of blood T cells from patients 1 and 2 at various times before and during the gene therapy trial. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (A) Production of IL-2 by cultured cells from patient 1 after stimulation with the mitogen PHA and with the specific antigens tetanus toxoid and influenza A virus as described (*30*). IL-2 was quantitated by bioassay measuring the proliferation of the IL-2–dependent T cell line CTLL at a 1:2 dilution of the lymphocyte culture supernatant. The fine dashed line indicates the patient's T cell count for reference. Solid triangles along the base line indicate the dates of cell infusion. (B) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell line and a ^{51}Cr-labeled allogeneic target B cell line by blood T cells from patient 1 as described (*31*). Lysis (as percent specific isotope release during a 6-hour incubation of effector and target cells at a ratio of 60:1) was measured after in vitro pre-stimulation for 7 days. Solid triangles along the base line indicate the dates of cell infusion. (C) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell and a ^{51}Cr-labeled allogeneic target B cell line by blood T cells from patient 2 as described above.

the mitogen phytohemagglutinin (PHA) (Fig. 4A) but were unable to produce IL-2 in response to stimulation with influenza A virus or tetanus toxoid, despite repeated immunization with these antigens. Over the first months of gene therapy, IL-2 production improved and became normal after 1 year (Fig. 4A). Again before gene therapy, patient 1's T cells failed to show significant cytolytic reactivity against either allogeneic cells or influenza A–infected target cells. Almost mirroring the steady increase in IL-2 production, she acquired normal in vitro cytolytic T cell responses to these antigens, reaching normal values in her second year of treatment. (Fig. 4B).

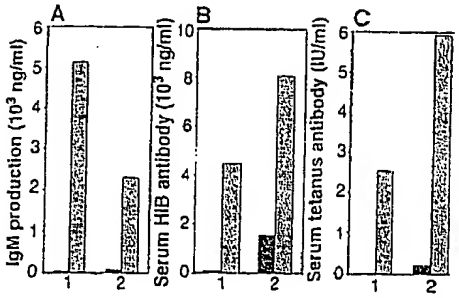
The results of these cytolytic assays for patient 2 are shown in Fig. 4C. Tests done 120 days before the beginning of gene therapy also showed impaired responses. However, cells that were obtained at the time of the first gene therapy infusion, cryopreserved, and subsequently tested some months later showed a normal cytolytic response to allogeneic cells. After a year on gene therapy, cytolytic T cell activity against influenza also became normal.

To evaluate the effects of our treatment on humoral immune function in these patients, we measured antibody responses to several antigens. Despite their PEG-ADA treatment, both patients 1 and 2 had only low or borderline titers of isohemagglutinins on repeated testing before gene therapy. Each patient showed significant elevations in the levels of these antibodies within 90 to 115 days of beginning treatment with gene-modified cells (Table 1). Isohemagglutinins are antibodies that react with group A and B red blood cell antigens and occur spontaneously as a result of environmental exposure to cross-reacting antigens. Isohemagglutinin responses are, therefore, less dependent on the timing of previous immunizations than are responses to common vaccine antigens. After gene therapy, each patient also had improvement in antibody responses to vaccines to *Hemophilus influenzae* B (HIB) and tetanus toxoid (Fig. 5). With enzyme therapy alone, peripheral lymphocytes from each patient were unable to produce immunoglobulin M (IgM) in vitro after stimulation with pokeweed mitogen (PWM), but made robust responses after a year on the gene therapy protocol (Fig. 5A). Immunoglobulin production to PWM depends on T cells; these results further confirm the reconstitution of T cell function associated with gene therapy.

The effects of this treatment on the clinical well-being of these patients is more difficult to quantitate. Patient 1, who had been kept in relative isolation in her home for her first 4 years, was enrolled in public kindergarten after 1 year on the protocol and has missed no more school because of infectious disease than her classmates or siblings. She has grown normally in height and weight and is considered to be normal by her parents. Patient 2 was regularly attending public school while receiving PEG-ADA treatment alone and has continued to do well clinically. Chronic sinusitis and headaches, which had been a recurring problem for several years, cleared completely a few months after initiation of the protocol.

This trial of retroviral-mediated gene transfer shows that the survival of reinfused transduced peripheral blood T cells is prolonged in vivo; the erroneous assumption that T cells would not have such long-term survival was often cited as a potential problem with this treatment strategy. Patient 1 has had a normal total peripheral T cell count since the last cell infusion, and the proportion of her circulating T cells carrying vector DNA has remained stable over that period. Further, expression of the ADA transgene under the influence of the retroviral long terminal repeat (LTR) promoter has persisted for a long period in vivo without obvious extinction. There have been swings in the level of ADA enzyme in her peripheral lymphocytes throughout the period of observation, but the level of blood ADA enzyme activity at 4 years (protocol day 1480) is equivalent to that found immediately after the last cell infusion 2 years earlier (Fig. 1A). Although the data have not yet been completely analyzed, blood obtained after 5 years showed continuation of this trend with, again, a normal T lymphocyte count and an equivalent ADA level.

The mechanism by which our treatment aided immune reconstitution in patient 2 is less clear. The responses of patient 2 to some in vitro immunologic tests were variable before beginning our treatment protocol, ranging from little or no detectable response to nearly normal responses on the blood sample from the day gene therapy began. This patient produced a normal antibody response to immunization with bacteriophage φX174 about a year before beginning gene therapy (8). Although we have shown several examples of depressed cellular and humoral immune responses that strongly improved after gene therapy, this highly variable immune reactivity while patient 2 was on PEG-ADA therapy alone complicates interpretation of the contribution of our therapy. There was a temporal relation between initiation of gene therapy and a normalized peripheral T cell count,



...umoral immune function of patients 1 and ...e (solid bars) and after (hatched bars) gene ...y. (A) IgM production by the patient's periph... blood mononuclear cells in cultures stimulated ... the T cell–dependent polyclonal activator ...M performed as described (*32*). "Before" sam... were from D(−9). Follow-up cultures were at D500 (patient 1) and D560 (patient 2). In each case, the patient's cells stimulated with the T cell–independent B cell stimulant EBV (*33*) produced normal amounts of IgM (not shown), indicating intact B cell function before and after gene therapy, as expected. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (B) Serum antibody response to *Hemophilus influenzae* B. Patient 1 had failed to respond to two immunizations while on PEG-ADA alone [D(−9) shown]. Her response at protocol D591 is shown, after immunization. Patient 2 had some HIB-specific antibodies present before therapy [D(−122)], whose amounts increased without additional immunization during the protocol (D560). (C) Serum tetanus antibody. Patient 1 had negligible response to five separate tetanus immunizations before gene therapy [D(−48) shown] but responded briskly at D731, 24 days after re-immunization. Serum titers for patient 2 are shown for D(−9), 140 days after immunization while on PEG-ADA alone, and after receiving gene therapy (D592), 32 days after a booster tetanus immunization.

improved DTH, appearance of tonsils and ...able lymph nodes, normalized isohemagglutinin response, and improved PWM response, as well as other factors. In view of the relatively low level of ADA gene transfer achieved in this patient, the potential contribution of the infusions of the culture-activated T cells to the patient's response must also be considered. Perhaps ex vivo T cell activation somehow bypassed a differentiation block that PEG-ADA alone was unable to relieve. Despite the low final percentage gene transfer achieved, a 1% level of ADA gene–corrected cells could represent 10^9 to 10^{10} ADA-expressing T cells distributed throughout the body that could readily contribute to immune improvement.

Since the beginning of the trial, the dose of PEG-ADA enzyme given to each of our patients has been decreased by more than half (patient 1, 14 U/kg/week; patient 2, 10 U/kg/week), during which time their immune function has improved. By contrast, worsened immune function has been seen in other ADA$^-$ SCID patients when their dose of enzyme has been similarly reduced (*10*, *23*). We do not want to expose these patients to the potential risk of recurrent immunodeficiency by completely stopping PEG-ADA enzyme treatment until we have better information about the quality and duration of the immune improvement achieved by this first-generation gene therapy trial. The role of continued exogenous enzyme treatment will be clarified here or in companion studies attempting stem cell gene correction (*24*).

The safety of retroviral-mediated gene transfer has been a central concern. At least in the short and intermediate term, no problems have appeared in any clinical trial using these vectors. In the longer term, the theoretical potential for retroviral vectors to cause insertional mutagenesis remains the primary concern. To date, there has been no indication that malignancy associated with this process will be a complication of retroviral-mediated gene transfer.

Our trial here has demonstrated the potential efficacy of using gene-corrected autologous cells for treatment of children with ADA$^-$ SCID. Eleven children with this disease have been enrolled in various gene therapy protocols, each using different strategies and retroviral vector designs and focusing on different target cell populations. The experience gained from these approaches should provide guidance for gene therapy as a treatment for this disorder as well as for a larger array of inherited and acquired diseases.

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21. Beginning with culture 9 for patient 1 and culture 5 for patient 2, the patients' lymphocyte populations obtained by apheresis were fractionated by adherence to flasks coated with CD8 monoclonal antibodies (Applied Immune Sciences) following the manufacturer's instructions. This protocol modification for CD8 depletion was introduced because both patients were developing a progressively inverted CD4-CD8 ratio. This effect was apparently the result of preferential growth of $CD8^+$ cells during the last 4 to 5 days of culture and the subsequent persistence of these infused $CD8^+$ cells in the circulation. Consequently, each subsequent apheresis sampled the recently increased number of $CD8^+$ cells, and thus the skewing of the ratio of CD4 to CD8 cells became compounded with each additional treatment. By partially depleting the apheresis sample of $CD8^+$ cells by an immunoaffinity selection process, the later treatments for each patient consisted of cells with a more balanced phenotype. The perturbation in normal CD4-CD8 cell proportions did not have detectable untoward effects for either patient.
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25. The ADA enzyme activity assay was performed in duplicate as described (*13*). Positive control cells were obtained from healthy normal donors and had a mean of 82 U (normal range, 66 to 102 U). Duplicate samples were run in the presence of the ADA enzyme inhibitor EHNA (30 μM). Specific ADA activity was calculated as total adenosine deaminating activity minus EHNA-resistant activity. EHNA-resistant activity represents metabolic activity of a nonspecific aminohydrolase present in human cells.
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28. Southern hybridization analysis for LASN vector consisted of the following: 10 μg of DNA was digested with Sst I and hybridized with a 728-bp Nco I fragment from LASN corresponding to the SV40 promoter and *neo* gene. DNA from K562-LASN cells served as a positive control.
29. RT-PCR analysis for LASN vector transcripts was as follows: 3 μg of polyadenylated RNA was treated with deoxyribonuclease and reverse-transcribed. The cDNA (0.3 μg) was amplified with LASN vector–specific primers in a 30-cycle PCR reaction. The oligonucleotides 5′-CAGCCTCTGCAGGGCAGAAC-3′ (corresponding to the 3′ end of the ADA gene in LASN) and 5′-GCCCAGTCATAGCCGAATAG-3′ (complementary to 5′ end of the *neo* gene in LASN) were used as primers. After electrophoresis and blotting, the sequences were hybridized with a 527-bp probe corresponding to the entire length of the predicted PCR product.
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Physical Map and Organization of *Arabidopsis thaliana* Chromosome 4

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A physical map of *Arabidopsis thaliana* chromosome 4 was constructed in yeast artificial chromosome clones and used to analyze the organization of the chromosome. Mapping of the nucleolar organizing region and the centromere integrated the physical and cytogenetic maps. Detailed comparison of physical with genetic distances showed that the frequency of recombination varied substantially, with relative hot and cold spots occurring along the whole chromosome. Eight repeated DNA sequence families were found in a complex arrangement across the centromeric region and nowhere else on the chromosome.

Arabidopsis thaliana has been adopted as a model organism for the analysis of complex plant processes by means of molecular genetic techniques (*1*). The increase in map-based cloning experiments makes the generation of a complete physical map of the *Arabidopsis* genome a high priority. In addition, the availability of such a map would enable the organization of the chromosome to be studied in more detail. Little is known about the organization of plant chromosomes, but the general picture is that of chromosomes carrying large numbers of dispersed [often retrotransposons (*2*)] and tandemly repeated DNA sequences (*3*). The relatively small (100 Mb) *Arabidopsis* genome has a much smaller number of repeated DNA sequences than do most other plant species; its five chromosomes contain ~10% highly repetitive and ~10% moderately repetitive DNA (*4*). The dispersion of most of these sequences among the low-copy DNA is unknown.

We discuss here a physical map, which we have presented on the World Wide Web (WWW) at URL: http://nasc.nott.ac.uk/JIC-contigs/JIC-contigs.html, of *Arabidopsis* chromosome 4, one of the two chromosomes carrying nucleolus organizing regions. The construction of this map allowed us to analyze the frequency of recombination along the whole chromosome, the integration of the physical with the cytogenetic map, the interspersion pattern of repeated and low-copy DNA sequences over the whole chromosome, and the arrangement of repeated DNA sequences over the centromeric region.

We generated the physical map by hybridizing probes to four yeast artificial chromosome (YAC) libraries (*5*), using colony hybridization experiments (*6*). The probes consisted of 112 markers genetically mapped to chromosome 4, 20 previously unmapped genes, random genomic fragments and sequences flanking transposable elements, and the 180–base pair (bp) repetitive element carried in pAL1 (*7*). Southern (DNA) blot analysis of YAC clones confirmed the colony hybridization results and revealed common restriction fragments in the different YAC clones hybridizing to a given marker. This demonstrated overlap between the inserts of the YAC clones. On the basis of these results, the YAC clones could be placed into 14 YAC contigs with a high degree of redundant YAC cover, ensuring an accurate map despite the presence of chimeric clones in the YAC libraries.

We generated YAC end fragments, using either inverse polymerase chain reaction (IPCR) or plasmid rescue (*8*), from YAC clones lying near the ends of each of the 14 contigs. The fragments were hybridized to Southern blots of YAC clones from adjacent contigs. In addition, YACs, as well as some of the end fragments generated by IPCR, were used to identify clones from a cosmid library of the Columbia ecotype (*9*). The cosmids were then used as new markers on the YAC libraries. These experiments reduced the number of contigs to four. In all but two instances, the end fragments revealed that the contigs were already overlapping. Experiments aimed at closing the last three gaps have been attempt-

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5. Polyadenylated [poly(A)$^+$] mRNA was prepared from total RNA with the use of Oligotex-dT resin (Qiagen). Reverse transcription (RT) reactions were carried out with a StrataScript RT-PCR kit (Stratagene) modified as follows: 50-μl reactions contained 0.1 μg/μl of *Arabidopsis* mRNA, 0.1 ng/μl of human AChR mRNA, 0.05 μg/μl of oligo(dT) (21-mer), 1× first strand buffer, 0.03 U/μl of ribonuclease block, 500 μM deoxyadenosine triphosphate (dATP), 500 μM deoxyguanosine triphosphate, 500 μM dTTP, 40 μM deoxycytosine triphosphate (dCTP), 40 μM fluorescein-12-dCTP (or lissamine-5-dCTP), and 0.03 U/μl of StrataScript reverse transcriptase. Reactions were incubated for 60 min at 37°C, precipitated with ethanol, and resuspended in 10 μl of TE (10 mM tris-HCl and 1 mM EDTA, pH 8.0). Samples were then heated for 3 min at 94°C and chilled on ice. The RNA was degraded by adding 0.25 μl of 10 N NaOH followed by a 10-min incubation at 37°C. The samples were neutralized by addition of 2.5 μl of 1 M tris-Cl (pH 8.0) and 0.25 μl of 10 N HCl and precipitated with ethanol. Pellets were washed with 70% ethanol, dried to completion in a speedvac, resuspended in 10 μl of H_2O, and reduced to 3.0 μl in a speedvac. Fluorescent nucleotide analogs were obtained from New England Nuclear (DuPont).
6. Hybridization reactions contained 1.0 μl of fluorescent cDNA synthesis product (5) and 1.0 μl of hybridization buffer [10× saline sodium citrate (SSC) and 0.2% SDS]. The 2.0-μl probe mixtures were aliquoted onto the microarray surface and covered with cover slips (12 mm round). Arrays were transferred to a hybridization chamber (3) and incubated for 18 hours at 65°C. Arrays were washed for 5 min at room temperature (25°C) in low-stringency wash buffer (1× SSC and 0.1% SDS), then for 10 min at room temperature in high-stringency wash buffer (0.1× SSC and 0.1% SDS). Arrays were scanned in 0.1× SSC with the use of a fluorescence laser-scanning device (3).
7. Samples of poly(A)$^+$ mRNA (4, 5) were spotted onto nylon membranes (Nytran) and crosslinked with ultraviolet light with the use of a Stratalinker 1800 (Stratagene). Probes were prepared by random priming with the use of a Prime-It II kit (Stratagene) in the presence of [^{32}P]dATP. Hybridizations were carried out according to the instructions of the manufacturer. Quantitation was performed on a PhosphorImager (Molecular Dynamics).
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Gene Therapy in Peripheral Blood Lymphocytes and Bone Marrow for ADA$^-$ Immunodeficient Patients

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Adenosine deaminase (ADA) deficiency results in severe combined immunodeficiency, the first genetic disorder treated by gene therapy. Two different retroviral vectors were used to transfer ex vivo the human ADA minigene into bone marrow cells and peripheral blood lymphocytes from two patients undergoing exogenous enzyme replacement therapy. After 2 years of treatment, long-term survival of T and B lymphocytes, marrow cells, and granulocytes expressing the transferred ADA gene was demonstrated and resulted in normalization of the immune repertoire and restoration of cellular and humoral immunity. After discontinuation of treatment, T lymphocytes, derived from transduced peripheral blood lymphocytes, were progressively replaced by marrow-derived T cells in both patients. These results indicate successful gene transfer into long-lasting progenitor cells, producing a functional multilineage progeny.

Severe combined immunodeficiency associated with inherited deficiency of ADA (*1*) is usually fatal unless affected children are kept in protective isolation or the immune system is reconstituted by bone marrow transplantation from a human leukocyte antigen (HLA)–identical sibling donor (*2*). This is the therapy of choice, although it is available only for a minority of patients. In recent years, other forms of therapy have been developed, including transplants from haploidentical donors (*3*, *4*), exogenous enzyme replacement (*5*), and somatic-cell gene therapy (*6*–*9*).

We previously reported a preclinical model in which ADA gene transfer and expression successfully restored immune functions in human ADA-deficient (ADA$^-$) peripheral blood lymphocytes (PBLs) in immunodeficient mice in vivo (*10*, *11*). On the basis of these preclinical results, the clinical application of gene therapy for the treatment of ADA$^-$ SCID (severe combined immunodeficiency disease) patients who previously failed exogenous enzyme replacement therapy was approved by our Institutional Ethical Committees and by the Italian National Committee for Bioethics (*12*). In addition to evaluating the safety and efficacy of the gene therapy procedure, the aim of the study was to define the relative role of PBLs and hematopoietic stem cells in the long-term reconstitution of immune functions after retroviral vector–mediated ADA gene transfer. For this purpose, two structurally identical vectors expressing the human ADA complementary DNA (cDNA), distinguishable by the presence of alternative restriction sites in a nonfunctional region of the viral long-terminal repeat (LTR), were used to transduce PBLs and bone marrow (BM) cells independently. This procedure allowed identification of the origin of

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EXHIBIT
3

and their progeny, after gene transfer. This combined therapy and marking strategy allowed us to investigate directly in humans some of the basic questions related to the potential of retroviral vectors for gene therapy in cells of the hemato-lymphopoietic lineages. Although gene transfer into human hematopoietic progenitors (*13, 14*), peripheral blood stem cells (*15*), and PBLs (*16–18*) has been extensively demonstrated in vitro, the potential for long-term survival in vivo after the manipulations required for retroviral vector gene transfer remains to be proven. In addition, this study allowed us to study the feasibility of gene transfer into hematopoietic stem and progenitor cells, and the potential for long-term persistence of differentiated cells in a context different from high-dose chemotherapy and BM transplantation (*19–21*). In this system, however, the positive selection may represent an absolute requirement for favoring the appearance of vector-transduced cells.

In ADA$^-$ patients, failure of the immune system to develop is due to the sensitivity of lymphocytes or their precursors to the toxic effects of accumulated ADA substrates (*22*). Because it is possible to reduce the levels of toxic metabolites in ADA$^-$ cells by providing exogenous ADA (*23*), a nonselective form of ADA replacement (that is, transfusion of irradiated red cells from normal individuals) has been used to treat ADA$^-$ patients (*24*). An improved form of treatment was developed by covalent attachment of polyethylene glycol (PEG) to the purified bovine enzyme (*23, 25*). PEGilation appears to block access of degradative enzymes, antibodies, and antigen-presenting cells to the protein surface, thereby inhibiting clearance from the circulation (*26–28*) and prolonging ADA plasma half-life from a few minutes to 24 hours (*23*). The main biochemical consequences of ADA deficiency are almost completely reversed by PEG-ADA treatment (*23*), resulting in an increase in circulating T lymphocytes and improvement of cellular immune functions (*23, 29*).

In our study, treatment in two patients [G.B., patient 1; A.R., patient 2 (*30*); both about 2 years of age] was initiated with weekly intramuscular injections of increasing doses of PEG-ADA (20 and 30 U per kilogram of body weight) until plasma ADA activity could be maintained at least in the normal range of total blood activity. The range of ADA activity was stable between 20 and 40 μmol hour^{-1} ml^{-1}. Before initiation of treatment, both patients had nearly undetectable intracellular ADA activity, and lymphopenia was observed in both patients. Approximately 50% of blood mononuclear cells reacted with monoclonal antibodies to T cell surface antigens, and their proliferative response to mitogens ranged from virtually undetectable to 10% of normal controls. Both patients showed some response in mixed lymphocyte culture, although they produced no specific antibody and showed no antigen-restricted T cell response. Residual immune functions were probably due to previous irradiated red cell transfusions. During the first year of PEG-ADA treatment, lymphocyte counts and proliferative responses to phytohemagglutinin (PHA) normalized.

Immunological reconstitution resulted in increased isohemoagglutinin titer and in cellular and antibody responses to vaccination with tetanus toxoid (TT) (*31*). Associated with reconstitution of immune functions was the complete reversion of all clinical signs of immunodeficiency. However, as reported elsewhere (*29*), the initial reconstitution in this case was limited by the failure to maintain PBL counts and, more markedly, antigen-specific and nonspecific proliferative responses. At that point, the two patients met the conditions that define PEG-ADA treatment failure, as reported in our approved clinical protocol (*12*). Failure of treatment was defined by an extensive number of laboratory parameters and immunological assays (*12*). In both patients, failure of treatment was observed in the absence of any acute illness or open infection episodes and was confirmed in three separate determinations. Waiting for potential recurrence of clinical symptoms such as infectious episodes, or failure of thriving, was considered to be inappropriate.

Early development of T cells obtained during PEG-ADA treatment was crucial to the implementation of the gene therapy protocol. Administration of PEG-ADA continued throughout the study period, although at decreasing amounts. Therefore, the relative role of gene-corrected cells and PEG-ADA treatment remains to be completely defined, an issue that will be addressed during the continuation of this study.

The aim of our study was to evaluate the safety and efficacy of the retroviral vector–mediated gene transfer procedure and to define the relative role of PBLs and BM stem and progenitor cells as effectors of long-term reconstitution of immune functions after gene transfer. For this purpose, we constructed two different retroviral vectors, DCA*l* and DCA*m*, expressing the human ADA cDNA under the control of its own promoter, which were used to infect PBLs and BM cells, respectively (Fig. 1). Both vectors are based on the double-copy (DC) design (*32*) and are structurally identical except for the presence of alternative restriction sites (Mlu I in DCA*l* and Bss HII in DCA*m*) in a nonfunctional region of the viral LTR (*33*). This feature allowed unequivocal tracing of the origin (BM or PBL) of the transduced cell progeny in the circulation by a simple polymerase chain reaction (PCR) analysis on genomic DNA (*34*). Both vectors were packaged in the amphotropic GP+*env* Am12 cell line (*33*). PBLs and T cell–depleted BM cells were transduced ex vivo either by multiple exposure to cell-free viral supernatant or by coculture with irradiated packaging cells (*35, 36*). Gene transfer efficiency increased from 1 to 2.5% up to 40% in total PBLs, with the introduction in the procedure of a new packaging line and of cocultivation. Gene transfer efficiency into CFU-GM and BFU-E hematopoietic progenitors averaged 30 to 40%, as described (*37*). These frequencies were estimated by cloning in lim-

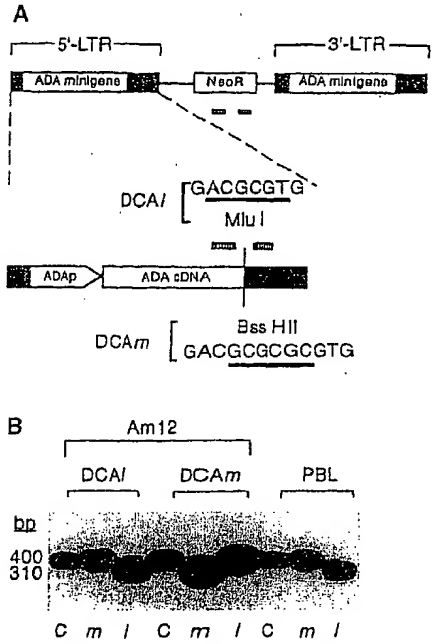


Fig. 1. (A) Structure of the DCA*l* (lymphocytes) and DCA*m* (marrow) proviruses. A human ADA minigene (promoter + full-length cDNA) was inserted into the LTR U3 region of a Moloney murine leukemia virus–derived retroviral vector (DCA) (*32, 33*). For construction of two vectors that could be distinguished from each other after integration into the target cell genome, the unique Mlu I restriction site present in a functionally irrelevant region of the LTR in DCA*l* was converted into a Bss HII site in DCA*m* (enlarged map). The hatched boxes indicate the location of the PCR primers used to detect vector DNA in target cells and for vector identification. (B) PCR identification of the vector integrated into the lymphocytes of patient 1 3 months after initial administration of DCA*l*-transduced PBLs and DCA*m*-transduced BM cells, showing the PBL origin of the transduced circulating lymphocytes. C, control (uncut PCR product); *m*, marrow-specific Bss HII cut present in DCA*m*-transduced cells; *l*, lymphocyte-specific Mlu I cut present in DCA*l*-transduced cells. PCR amplification of DNA obtained from the DCA*l* and DCA*m* packaging cell lines (Am12) is shown as a control.

dilution (38) and semi-solid colony-forming assays (39), respectively, in the presence or absence of G418 and are the result of the steady improvement in both cell-free infection and cocultivation that we have produced in recent years (*10*, *11*, *18*, *37*). In particular, our goal has been to increase gene transfer frequency while maintaining phenotype, immune repertoire, and in vivo potential for proliferation, differentiation, and survival. For this purpose, short cultivation time under conditions of low interleukin-2 (IL-2) concentration were developed for the activation and infection of PBLs (*35*), while BM cells were maintained in a long-term culture system over adherent layers without addition of exogenous growth factors, and were infected during the first 3 days of culture (*36*). This system produces minimal loss of differentiation capacity and potential for in vivo hematopoietic reconstitution (*40*). No G418 selection was applied to infected PBLs or BM cells before reinfusion. Transduction efficiency and production of the vector-derived ADA in infected cells was determined by PCR and thin-layer chromatography (TLC), respectively (*41*).

In vivo administration of genetically modified cells began in March 1992 for patient 1 and July 1993 for patient 2. Patient 1 received 7.24×10^8 DCA*I*-transduced lymphocytes and 0.35×10^8 DCA*m*-transduced progenitor cells in nine injections administered intravenously (i.v.) over a period of 24 months. Patient 2 received a slightly smaller number of cells in five injections over 10 months.

We began monitoring the persistence of vector-transduced cells at monthly (or bimonthly) intervals from the first infusion. Analyses were performed both on bulk populations of cells of different origin, for the indication of origin of transduced cells, and on clonal assays for quantitation of transduced BM and PBLs. Six months after the beginning of treatment, long-term survival of transduced cells was demonstrated in both patients by the presence of vector-derived sequences in the DNA extracted from peripheral blood mononuclear cells, total BM cells, mature granulocytes (Fig. 2A), individual T lymphocyte clones (Fig. 3), and BM progenitors (BFU-E, CFU-GM, and CFU-GEMM) in clonal culture (*41*). ADA production at levels substantially greater than observed in untransduced ADA$^-$ controls was observed in PBLs, BM, and granulocytes (Fig. 2C). The proportion of genetically modified cells in BM and circulating blood was monitored throughout the study by BM and T cell clonal assay in the presence of G418, and indirectly from the amount of ADA activity in total cell populations from BM and peripheral blood. In both patients, this proportion ranged between 5 and 30% of clonable BM progenitors and between 0.8 and 8.5% in PBLs. Total ADA activity ranged between 5 and 18% of normal values in both patients' nucleated cells in the blood. Sixteen months after discontinuation of treatment, ADA activity in total circulating nucleated cells was 126 nmol hour^{-1} per milligram of protein in patient 1 and 77 nmol hour^{-1} per milligram of protein in patient 2 (internal normal control, 1157 nmol hour^{-1} per milligram of protein; for method see legend to Fig. 2). At the same time, in patient 1, the frequency of transduced G418-resistant T cell was 4.76% and that of clonable BM progenitors was 25%; in patient 2 these frequencies were 2.01 and 17%, respectively. During this period in patient 1, and more recently in patient 2, ADA activity became reproducibly detectable also in circulating erythrocytes (Fig. 2C). Vector-derived ADA activity in individual T cell clones was comparable to, or higher than, that of normal controls (legend to Fig. 2C), as observed in T cells that survived in vivo selection in the human PBL-SCID mouse preclinical model (*10*, *11*). ADA activity in Neo-resistant BM colonies also averaged normal levels (*41*).

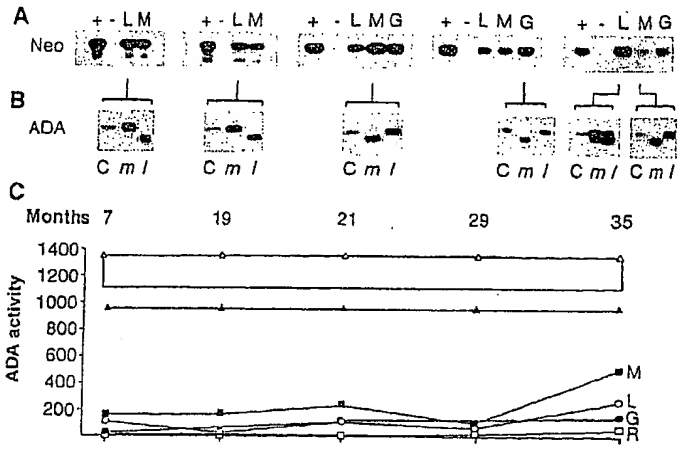


Fig. 2. Persistence of transduced hematopoietic cells in vivo and analysis of their origin. During 3 years after initiation of the gene therapy trial, persistence of transduced PBLs and BM cells, and expression of vector-derived ADA activity were documented at regular intervals. (**A**) Detection of transduced cells by PCR analysis for the NeoR gene was consistent throughout the follow-up of patient 1 in PBLs (L), BM cells (M), and circulating granulocytes (G). + and −, PCR positive and negative controls, respectively. (**B**) Analysis of the identity of the integrated vector showed that vector-positive lymphocytes were initially all derived from long-lived transduced PBLs, as demonstrated by the presence of the DCA*I*-specific PCR pattern (7 and 19 months), whereas BM and granulocytes showed the DCA*m*-specific pattern (21, 29, and 35 months). Three years after initiation and 1 year after discontinuation of treatment, DCA*m*-specific signals started to appear in the DNA extracted from PBLs, indicating progressive conversion of the circulating, genetically modified lymphocyte pool from a predominantly PBL-derived to a BM progenitor–derived population (35 months). This observation was further confirmed by the analysis of Neo-resistant, peripheral blood T cell clones (Fig. 3). (**C**) In parallel, vector-derived ADA activity was monitored by TLC in total PBLs (L) and BM cells (M) of patient 1. G, granulocytes; R, red blood cells. Two positive controls are provided: ADA activity (mean ± SE) from a pool of normal individuals (△), and ADA activity from a polyclonal PBL line from the same patient transduced in vitro and selected in G418 (▲). Lysates were prepared from 1×10^6 cells in 10 μl of CGLB buffer by three cycles of freeze and thaw. ADA enzyme activity was analyzed by the ^{14}C-adenosine to ^{14}C-inosine conversion assay followed by TLC (*37*). Cell lysates from individual clones (~2×10^5 cells) were normalized for protein content by the BIO-RAD protein assay (Bio-Rad Laboratories GmbH, Munich, Germany). Positive and negative controls were, respectively, lysates from normal PBLs and uninfected, IL-2–stimulated ADA$^-$ PBLs, because IL-2 stimulation is reported to increase the efficiency of ADA expression in ADA$^-$ cells (*37*). TLC plates were exposed for 3 days in a PhosphorImager (Molecular Dynamics, Sunnyvale, California). Ratio of adenosine conversion is expressed as nmol hour^{-1} mg^{-1}.

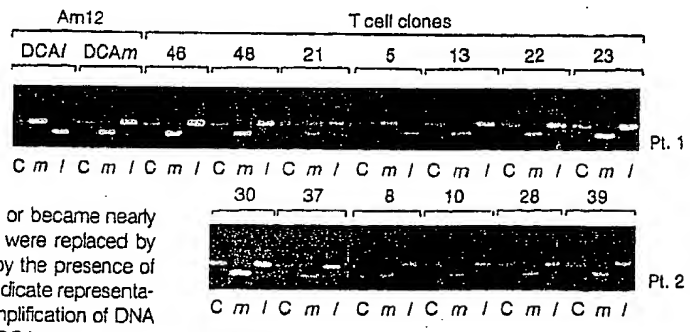


Fig. 3. Origin of T cell clones obtained from the peripheral blood of patient 1 (top) and patient 2 (bottom) 1 year after discontinuation of treatment. Clonable T cells containing the DCA*I* vector diminished markedly (patient 1) or became nearly undetectable (patient 2), and were replaced by BM-derived T cells, marked by the presence of the DCA*m* vector. Numbers indicate representative individual clones. PCR amplification of DNA obtained from the DCA*I* and DCA*m* packaging cell lines (Am12) is shown as a control.

taneous revertants (ADA-positive, -negative) were not observed in either peripheral blood or BM.

Initially, the analysis of the retroviral vector amplified from the DNA of circulating lymphocytes indicated that genetically modified cells were derived from a pool of long-lived PBLs originally transduced with the DCA*l* vector (Fig. 1B). This finding was consistent throughout the period of administration of transduced PBLs and BM cells (Fig. 2B, at 7 and 19 months, for example), whereas total BM cells (Fig. 2B, at 21 and 35 months, for example) and circulating granulocytes (Fig. 2B, at 29 months, for example) always showed the DCA*m*-specific restriction pattern or marrow-derived cells. However, about 1 year after discontinuation of gene therapy, both PBL- and BM-derived lymphocytes were detectable in the circulation (Fig. 2B, at 35 months). At first time, Neo-resistant, clonable T cells containing the PBL-specific DCA*l* vector sharply decreased (Fig. 3, patient 1) or became undetectable (Fig. 3, patient 2) and were progressively replaced in the circulation by T cells containing the BM-specific, DCA*m* vector. To confirm this important finding, we evaluated two additional time points, subsequent to the data in Figs. 2 and 3, on bulk populations and on T lymphocyte clones. Thirty-eight clones from patient 1 were analyzed for their origin; 26 were derived from marrow, 6 could not be unequivocally determined, and 9 contained the DCA*l* vector. Similarly, of 49 clones obtained from patient 2, 6 could not be clearly determined, 6 contained the DCA*l* vector, and all others were derived from marrow.

These results show that short-term immune reconstitution was sustained in the two patients by a population of peripheral blood–derived, ADA-producing lymphocytes with a life-span in the circulation ranging between 6 and 12 months. We have previously shown that this population contains both mature T cells and immature, or naïve, precursors (*11*, *18*). Conversely, long-term reconstitution resulted almost exclusively from transduced, BM-derived hematopoietic stem and progenitor cells capable of generating multilineage progenies of ADA-producing cells, that is, lymphocytes, granulocytes, and (more recently) erythrocytes.

A fundamental hypothesis underlying this study was the possibility that genetically corrected cells would benefit from a selective advantage over noncorrected cells. Our experimental design has made it possible to obtain data in support of this hypothesis. The first line of evidence comes from the progressive appearance of marrow-derived PBLs, generated over time from a relatively small number of genetically modified precursors contained in the transduced marrow cell population (Figs. 2 and 3). Additional evidence comes from the analysis of the integrated retroviral vectors. In a recent comparative analysis of different vector constructs designed for gene transfer of reporter genes in human PBLs, we demonstrated that the DC construct carries an inherent instability that results in loss of the gene inserted in the viral LTR (*18*). Such instability could affect 50% of integrated proviruses, depending on the size and nature of the inserted gene. In the present study, the analysis of over 200 T cell clones obtained at different times during the follow-up of the two patients showed no rearrangement that might have eliminated the ADA gene, and consequently its expression. Conversely, loss of the ADA gene could be detected only in marrow-derived colonies and T cell clones that had been transduced and cultured in vitro, in the absence of any positive selection (*41*). These observations indicate that, in ADA$^-$ SCID patients, ADA-producing cells have a selective advantage over noncorrected ADA$^-$ cells, as previously suggested in the

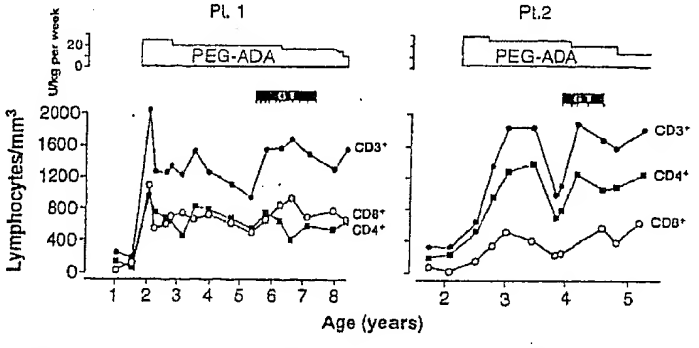


Fig. 4. Immune reconstitution during the PEG-ADA and gene therapy trial for patient 1 (left) and patient 2 (right). CD3$^+$, CD4$^+$, and CD8$^+$ lymphocyte counts are plotted against age for the duration of the trial. Doses of PEG-ADA administered to the patients are shown in the upper part of the graphs. The black boxes (GT) indicate the period of administration of genetically modified PBLs and BM cells (ticks indicate individual injections).

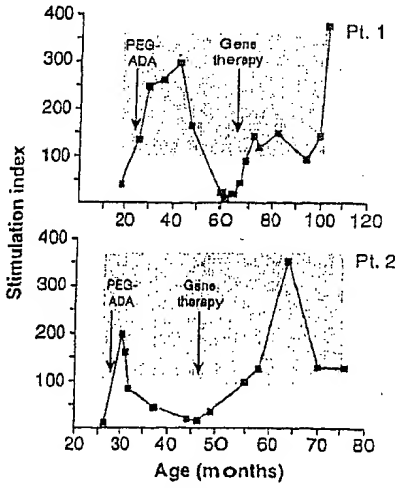
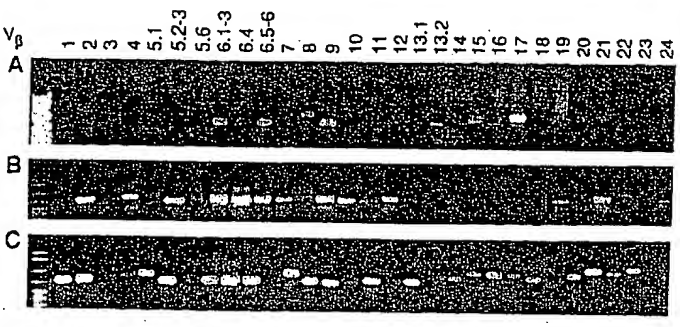


Fig. 5. Immune reconstitution during the PEG-ADA and gene therapy trial for patient 1 (top) and patient 2 (bottom). T cell proliferative response to mitogenic stimulus is presented as stimulation index (cpm of stimulated samples divided by cpm of unstimulated cells) and is plotted against age of the patients. Shaded areas indicate the range of the stimulation index of normal internal controls. Response to TT followed a comparable kinetics. Arrows indicate initiation of enzyme replacement therapy (PEG-ADA) and administration of genetically modified cells (gene therapy).

Table 1. Quantitation of isoagglutinin titer, and antigen-specific antibody production and proliferative response after vaccination with tetanus toxoid (TT). IgG, immunoglobulin G; ND, not done.

Time of test	Anti–B isoagglutinin titer		Serum titer of anti-TT IgG*		Proliferative response (10^3 cpm)†	
	Patient 1	Patient 2	Patient 1	Patient 2	Patient 1	Patient 2
Before immunization	ND	1/2	2.4	0.2	0.5	1.6
PEG-ADA response	1/8	1/16	1600	130	26.4	15.7
PEG-ADA failure	1.8	1.5	ND	34	1.8	1.5
After gene therapy I	1/16	1/32	ND	88	50.3	67.6
After gene therapy II	1/32	1/32	ND	ND	82.2	96.5

*Anti-TT IgG production was determined in a standard enzyme-linked immunosorbent assay and is reported as international units to a reference standard (Biagini Reference Standard, Biagini, Florence, Italy). †TT-specific T cell lines were tested for their capacity to proliferate in response to TT in the presence of autologous antigen-presenting cells (*45*). Proliferation in response to antigen-presenting cells alone was always <1000 cpm. Both patients received the full immunization schedule with TT (three doses) while on PEG-ADA. Patient 2 showed signs of immune deterioration during the immunization schedule. Isoagglutinin titer, TT-specific IgG titer, and TT-specific T-cell proliferation were measured at the following times: before immunization and before use of PEG-ADA (2 years and 5 months of age for patient 1 and 2 years and 10 months for patient 2), at the time of peak response to PEG-ADA (2 years since the beginning of PEG-ADA treatment for patient 1 and 1 year for patient 2), at PEG-ADA failure, and twice after gene therapy (6 and 8 years of age for patient 1 and 4.5 years and 5 years for patient 2).



...evelopment of a ...T cell receptor ...oire in patient 1 af... ...ene therapy treat... ...nt. Different T cell re... ...ptor V_β-chain usage at ...e time of failure of the ...EG-ADA treatment (A), ...1 year after the begin... ...ning of gene therapy (B), and 1 year after discon... ...tinuation of transduced cell administration (C), as analyzed by RT-PCR amplification with V_β chain-specific primers (*48*).

human PBL–SCID mouse preclinical model (*10*, *11*).

Immune reconstitution induced by PEG-ADA treatment lasted for over 3 years in patient 1 and for a shorter period in patient 2, despite administration of a 50% higher PEG-ADA dose in the latter (Figs. 4 and 5). In association with a progressive decline in PBL counts, the immune response decreased markedly over a short period of time (Figs. 4 and 5 and Table 1). Administration of genetically modified cells rapidly restored immune functions in both patients, resulting in normalization of total lymphocyte counts (Fig. 4) and cellular and humoral responses, including sustained isohemoagglutinin titer, antigen-specific antibody production, and mitogen- and antigen-specific proliferation (Fig. 5 and Table 1). The T cell receptor repertoire, analyzed by the V_β chain usage, normalized progressively (Fig. 6). In patient 2, the overall response to gene therapy was similar to that of patient 1, despite administration of a smaller number of genetically modified PBLs and BM cells. There have been no serious infections in the two patients throughout the PEG-ADA treatment and after the beginning of gene therapy. The patients received no other treatment, except for high-dose immunoglobulins administered intravenously and prophylactic antibiotic treatment, both of which were discontinued after indications of full immunologic reconstitution. Before the beginning of the PEG-ADA treatment, the patients showed severe growth failure, ranging below the fifth percentile for height and weight. Enzyme replacement and gene therapy had a marked clinical impact, resulting in normalization of height and weight. Patient 2, who had a very limited initial response to PEG-ADA, resumed normal growth only after gene therapy. Serum chemistry values, blood counts, and urinalysis indicated no toxicity from PEG-ADA or gene therapy treatments. Monitoring of the two patients for the presence of recombinant helper virus was consistently negative.

The results of the long-term follow-up have two main implications: the selection of an optimal treatment for ADA$^-$ SCID patients and, more generally, the potential application of similar gene therapy approaches to the treatment of genetic and acquired diseases. Our study clearly indicates the feasibility of direct BM cell gene therapy; however, in specific circumstances, genetically modified PBLs may provide a prompt supply of immune effector cells until development of BM-derived lymphocytes. If it is proven to be efficacious over time, this procedure could represent a less toxic alternative to unrelated or HLA-mismatched marrow transplants. In the prospective extension of these results to the design of other gene therapy clinical trials, gene transfer into hematopoietic progenitors can be achieved also in the absence of the stress conditions associated with cytoreduction and BM transplantation. However, in steady-state hematopoiesis a considerable time lag may be required before appearance of genetically modified cells in the blood. Under these conditions, the positive selection may represent an absolute requirement for favoring the appearance of vector-transduced cells. Such positive selection may be "naturally" present in other genetic or acquired diseases [for example, acquired immunodeficiency syndrome (AIDS)] or could be built into the vector as a drug-resistance gene.

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31. Both patients were vaccinated with tetanus toxoid (TT; Swiss Serum Institute, Bern, Switzerland). To evaluate the number of precursor lymphocytes specific to TT, the frequency of cells capable of proliferating in the presence of irradiated autologous PBLs that had been pulsed overnight with TT was evaluated in a limiting dilution assay. At two different times after vaccination, the frequency of TT-specific lymphocyte precursors was 1:2500 and 1:5000 in patient 1. This range is comparable to that of normal individuals at the same time after immunization. Patient 2 showed signs of immune deterioration during the immunization schedule (Table 1).
32. P. A. Hantzopoulos, B. A. Sullenger, G. Ungers, E. Gilboa, *Proc. Natl. Acad. Sci. U.S.A.* 86, 3519 (1989).
33. The double-copy DCA retroviral vector, carrying a human ADA minigene, has been described previously (*32*) and is indicated as DCA*I* in this report. The DCA*m* vector was obtained by digestion, Klenow-filling, and religation of the DCA*I* plasmid at the Mlu I restriction site in the polylinker region of the 3′ LTR, thus generating a new Bss HII site. The Am12/DCA*m*-7 and the Am12/DCA*I*-18 clonal packaging cell lines were generated by the transinfection protocol, as described (*18*).
34. High molecular weight DNA was obtained from cells by standard phenol-chloroform extraction (*42*). A Neo-specific, 362-bp fragment located in the coding region of the NeoR gene or a DCA-specific 400-bp fragment spanning the 3′ end of the ADA cDNA, the polylinker, and part of the LTR U3 region, were amplified from 0.5 μg of genomic DNA by 30 cycles of PCR with 5 U of Taq polymerase (Perkin Elmer, Norwalk, CT) and 25 pmol of the primers Neo-1 (5′-GGAAGCCGGTCTTGTCGATC-3′), Neo-3 (5′-AGAGTCCCGCTCAGAAGAAC-3′), DCA-5 (5′-TCAATGCGGCCAAATCTAG-3′), and DCA-6 (5′-GCTGTTCCATCTGTTCCTGA-3′). DCA-specific PCR products were digested by Bss HII and Mlu I restriction enzymes (Boehringer Mannheim GmbH, Mannheim, Germany). Reaction mixtures (1/10th of the total volume) were separated on a 1.5% agarose gel, and DNA was visualized by ethidium bromide staining. DNA was transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) by DNA capillary blotting (*42*) and hybridized to 10^7 dpm of ^{32}P-labeled, 1.2-kb Hind III–Sma I fragment of pSV2-neo (*43*), or 1.9 Kb Xho I fragment of the ADA cDNA. Filters were washed under high-stringency conditions and exposed to Kodak X-AR5 film for 30 min to overnight at −70°C.
35. Freshly isolated ADA$^-$ PBLs were obtained from ADA$^-$ SCID patients, Ficoll-fractionated, and grown at 10^6 cells/ml in 24-well tissue culture plates under phytohemagglutinin (PHA) (2 μg/ml; Boehringer Mannheim GmbH) and human recombinant IL-2 (hu-rIL-2, 100 U/ml; Eurocetus B.V., Amsterdam, Netherlands) stimulation in lipopolysaccharide-free RPMI 1640 medium supplemented with 2 mM L-glutamine and 5% human serum. In subsequent experiments similar levels of gene transfer could be obtained at 50 or 100 U of hu-rIL-2 per milliliter, in the absence of any additional stimulus (C. Benati *et al.*, in preparation). After 72 to 96 hours of stimulation, T lymphocytes were cocultivated with irradiated (10,000 roentgen) vector-producing cells for 72 hours in complete Dul-

's minimum essential medium (DMEM) in the ence of polybrene (8 μg/ml) as described (*18*). were washed twice in phosphate-buffered saline S), resuspended in fresh medium, and cultured for to 4 days. Transduced cells were tested for the presence of helper virus and cryopreserved until use. BM mononuclear cells were obtained as a Ficoll fraction and grown for 2 to 3 days in complete DMEM at a density of 6×10^5 to 8×10^5 cells/cm^2 (*35*). T cell depletion and progenitor cell enrichment were obtained as described (*3*, *35*). Gene transfer was carried out by multiple infection cycles with cell-free, helper virus–tested viral supernatants in the presence of polybrene (8 μg/ml) (*35*). BM cells were maintained in a long-term culture system over adherent layers without addition of exogenous growth factors, and infected during the first 3 days of culture. Transduced cells were tested for the presence of helper virus and cryopreserved until use. At that time, the transduced cells were washed, resuspended in normal saline containing 4% human albumin, and reinfused into the patient.

37. C. Bordignon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86, 6748 (1989).
38. PHA blasts or antigen-specific T cells were cloned by limiting dilution. The relative frequencies of transduced cells was obtained by comparing the precursor frequency in the absence and presence of G418 (800 μg/ml). G418-resistant T cell clones were isolated and maintained as described (*44*, *45*).
39. The relative frequencies of transduced BM progenitor cells were obtained by comparing the frequency of CFU-G, CFU-GM, BFU-E, and CFU-GEMM cells in the absence and presence of increasing doses of G418 (0.7, 1.0, 1.5 mg/ml) as described (*37*). In selected experiments, individual G418-resistant colonies were collected for analysis of vector transduction and expression.
40. M. J. Bamett *et al.*, *Blood* 84, 724 (1994).
41. C. Bordignon *et al.*, data not shown.
42. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989).
43. P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* 1, 327 (1982).
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47. E. Y. Loh, J. F. Elliot, S. Cwirla, L. L. Lanier, M. M. Davis, *Science* 243, 217 (1989).
48. T cell receptor V_β-chain usage was analyzed on transduced T cell lines by reverse transcriptase–PCR. Briefly, total RNA was reverse transcribed with oligo(dT) and oligo(dG) primers and subjected to PCR with V_β- or C_β-specific oligonucleotides (*46*) or to anchored PCR with a C_β-specific oligonucleotide as described (*47*). Amplified products were analyzed by agarose gel electrophoresis.
49. We are indebted to L. Ruggieri and A. Wack for performing some of the ex vivo and in vitro analyses of gene transfer frequency; to the nurses and clinical staff of the Clinica Pediatrica, School of Medicine, University of Brescia, for skilled and dedicated care; to A. Arrighini and A. Crescenzo for clinical assistance in the extended care of the two patients; to A. Plebani for dosing specific antibody production; to M. Hershfield, P. Dellabona, and A. Ballabio for helpful discussions; and to Enzon, Inc., and Ophan Europe for providing PEG-ADA before commercial distribution. Supported by grants from Telethon, the Italian National Research Council, and the Italian Ministry of Health (IV-VII AIDS Projects).

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T Lymphocyte–Directed Gene Therapy for ADA$^-$ SCID: Initial Trial Results After 4 Years

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In 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA$^-$ SCID). The number of blood T cells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.

The possibility of using gene transfer as a therapy for human disease has great appeal. The decision to enter clinical trials awaited the development of safe and efficient techniques of gene transfer and improved understanding of the basic pathology and biology underlying likely candidate diseases and target cells. The advent of useful retroviral vectors that permitted relatively high efficiency gene transfer and stable integration was a critical advance (*1*, *2*), as was the demonstration that this procedure of gene transfer could be effectively and safely used in humans (*3*).

Severe combined immunodeficiency secondary to a genetic defect in the purine catabolic enzyme adenosine deaminase [ADA$^-$ SCID] is characterized by defective T and B cell function and recurrent infections, often involving opportunistic pathogens. Large amounts of deoxyadenosine, an ADA substrate, are present in these patients; deoxyadenosine is preferentially converted to the toxic compound deoxyadenosine triphosphate in T cells, disabling the immune system (*4*).

Because this disease is curable by allogeneic bone marrow transplantation given without pretransplantation cytoreductive conditioning, it was initially assumed that gene therapy should be directed at the bone marrow stem cell. However, initial attempts to use stem cell gene transfer in primates resulted in only low-level, transient gene expression, insufficient for clinical use. The observation that the only donor cells detected in some patients "cured" by allogeneic bone marrow transplantation was their T cells—the others remaining ADA-deficient (*5*)—raised the possibility that T cell–directed gene therapy also might be a useful treatment.

The introduction of enzyme replacement with ADA-containing erythrocytes (*6*) or with bovine ADA conjugated with polyethylene glycol (PEG-ADA) (*7*) has made this approach feasible. PEG-ADA has provided noncurative, life-saving treatment for ADA$^-$ SCID patients; with this treatment, most patients have experienced weight gain and decreased opportunistic infections. Full immune reconstitution has been less regularly achieved with enzyme therapy. T cell function as measured by in vitro mitogen responses improved in most patients, but fewer patients recovered consistent immune responses to specific antigens [for instance, as measured by normal delayed-type hypersensitivity (DTH) skin test reactivity] (*8–10*). Nearly all PEG-ADA–treated patients showed increased peripheral T cell counts, which provided a source of T cells for gene correction not available without enzyme therapy. Furthermore, enzyme treatment could be continued during the gene therapy trial so that the ethical dilemma of withholding or stopping a life-saving therapy to test an unknown treatment could be avoided.

The adenosine deaminase complementary DNA (cDNA) (*11*) is 1.5 kb and fits within a retroviral vector. With the use of an ADA-containing retroviral vector, ADA-deficient T cell lines were transduced to express normal amounts of ADA; this rendered them normally resistant to intoxication and growth inhibition when challenged with deoxyadenosine (*12*, *13*). Next, studies in mice, rabbits, and nonhuman primates using T cells modified with retroviral vectors showed normal cell survival and function after their reintroduction into recipient animals (*14*). Finally, Bordignon and colleagues (*15*) showed that ADA gene–corrected T cells acquired a survival advantage compared with uncorrected ADA-deficient cells when transplanted into immunodeficient, but ADA-

Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia

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An *ex vivo* approach to gene therapy for familial hypercholesterolaemia (FH) has been developed in which the recipient is transplanted with autologous hepatocytes that are genetically corrected with recombinant retroviruses carrying the LDL receptor. We describe the treatment of a 29 year old woman with homozygous FH by *ex vivo* gene therapy directed to liver. She tolerated the procedures well and *in situ* hybridization of liver tissue four months after therapy revealed evidence for engraftment of transgene expressing cells. The patient's LDL/HDL ratio declined from 10–13 before gene therapy to 5–8 following gene therapy, improvements which have remained stable for the duration of the treatment (18 months). This represents the first report of human gene therapy in which stable correction of a therapeutic endpoint has been achieved.

Familial hypercholesterolaemia (FH) has emerged as an important model for the development of human gene therapies[1]. This disorder, caused by inherited deficiency of LDL receptors, is associated with severe hypercholesterolaemia and premature coronary artery disease[1]. The homozygous form of FH is an excellent candidate for early applications of gene therapy because it is a lethal disorder that is refractory to conventional therapies. Measurement of serum lipid profiles provides a convenient and clinically relevant endpoint to evaluate response to therapy, and orthotopic liver transplantation has been shown to correct the underlying dyslipidemia indicating that hepatic reconstitution of LDL receptor expression is sufficient for metabolic correction[2,3].

The original paradigm for liver-directed gene therapy was based on transplantation of autologous hepatocytes genetically modified *ex vivo* with recombinant retroviruses. The efficacy and safety of this approach for treatment of FH has been demonstrated in a variety of animal models. A strain of rabbits genetically deficient in LDL receptors, called the Watanabe Heritable Hyperlipidemic (WHHL) rabbit, was used to demonstrate the potential efficacy of *ex vivo* gene therapy. Analysis of recipient animals demonstrated stable engraftment of genetically modified hepatocytes and persistent reductions in serum cholesterol for the duration of the experiment — 6.5 months[4]. Experiments in larger animals including dogs and baboons documented the feasibility and safety of *ex vivo* gene therapy directed to the liver[5,6]. *In situ* hybridization analysis of liver tissue from baboons harvested 1.5 years after gene therapy demonstrated stable engraftment of transgene expressing hepatocytes, providing further support for the efficacy of this therapy (unpublished data).

Based on the encouraging results obtained in animal models, we proposed a clinical protocol to treat FH homozygous patients with *ex vivo* gene therapy. We received permission from the US Recombinant DNA Advisory Committee (RAC) and the US Food and Drug Administration to treat three patients who had developed overt coronary artery disease and therefore would have a poor prognosis. Our experience with the first patient, described here, supports the efficacy and safety of liver-directed *ex vivo* gene therapy in humans.

First recipient of liver-directed gene therapy

The first clinical application of liver-directed gene therapy in humans used the *ex vivo* approach in a patient with homozygous FH. Although we were allowed to treat FH patients of any age, the RAC suggested that we enroll an adult as the initial patient to simplify the informed consent process. Patient FH1 underwent gene therapy on June 5, 1992. This French Canadian woman, who at the time of gene therapy was 28 years old, had a myocardial infarction at the age of 16 and required coronary artery bypass at the age of 26. Her dyslipidemia — which at baseline included a total serum cholesterol concentration of 545 mg dl^{-1}, LDL of 482 mg dl^{-1} and HDL of 43 mg dl^{-1} — was refractory to treatment with a variety of drugs including HMG CoA reductase inhibitors and bile acid binding resins. Genotype analysis indicated she was homozygous for a missense mutation (Trp66Gly, exon 3) that renders the LDL receptor incapable of binding to its ligands[7]. Cardiac evaluation performed prior to gene therapy revealed failure of one of her grafts and diffuse disease in her native coronary arteries, however, she was not overtly symptomatic.

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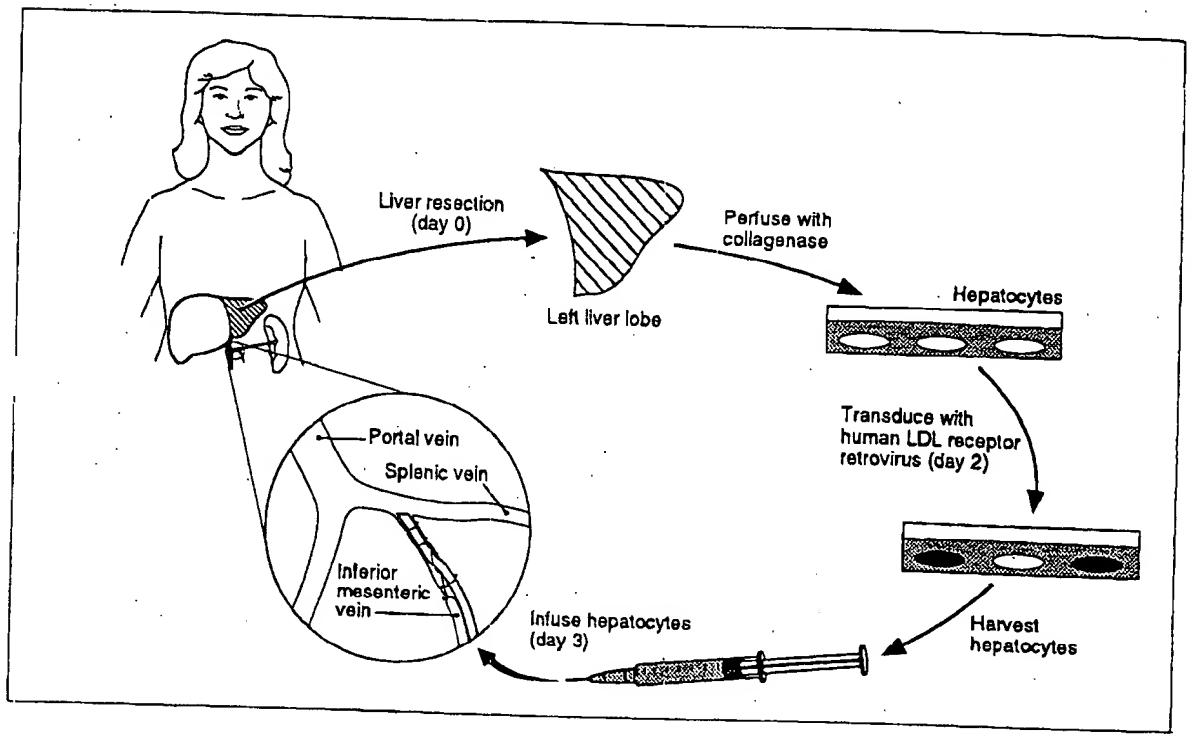

Fig. 1 Strategy of *ex vivo* gene therapy for familial hypercholesterolaemia.

Ex vivo gene therapy to liver is feasible and safe

The clinical protocol approved by the RAC has been published[8]; the general strategy is summarized in Fig. 1. The left lateral segment of the patient's liver, comprising approximately 15% of its total mass, was removed through a left subcostal incision. A 9.6 fr Hickman catheter was inserted into her inferior mesenteric vein, and the distal end of the catheter was brought through her incision thereby providing convenient access to the portal circulation for subsequent cell infusions. The resected liver, weighing 250 g, was perfused with collagenase to release hepatocytes; 3.2×10^9 cells were recovered (98% viability) and seeded into 800 10 cm^2 plates. Medium containing the LDL receptor expressing recombinant retroviruses was placed onto the cultured hepatocytes 48 hours after the initial seeding. Following a 12–18 h exposure to virus, the cells were analysed for LDL receptor expression and harvested for transplantation; 2×10^9 viable cells were recovered from the plates by treatment with trypsin. Incubation of the transduced cells with fluorescent labelled LDL revealed uptake in approximately 20% of the cells exposed to the LDL receptor expressing virus and no uptake in duplicate plates of cells not exposed to virus (Fig. 2).

Prior to infusion of the cells, a portal venogram was performed to confirm the placement of the catheter and patency of the portal circulation (Fig. 3*a*). The genetically corrected hepatocytes were harvested in three aliquots and each aliquot was manually infused at 4 h intervals directly into the catheter over a 30 minute period (a rate of ~2 cc min^{-1}). During the cell infusions the patient was carefully monitored in the intensive care unit; her vital signs measured during this time are presented in Fig. 4. She tolerated the cell infusions well except for a transient tachycardia early in the day, thought to be secondary to anxiety, and fevers that were present before cell infusion which resolved subsequently.

One concern was the potential development of portal

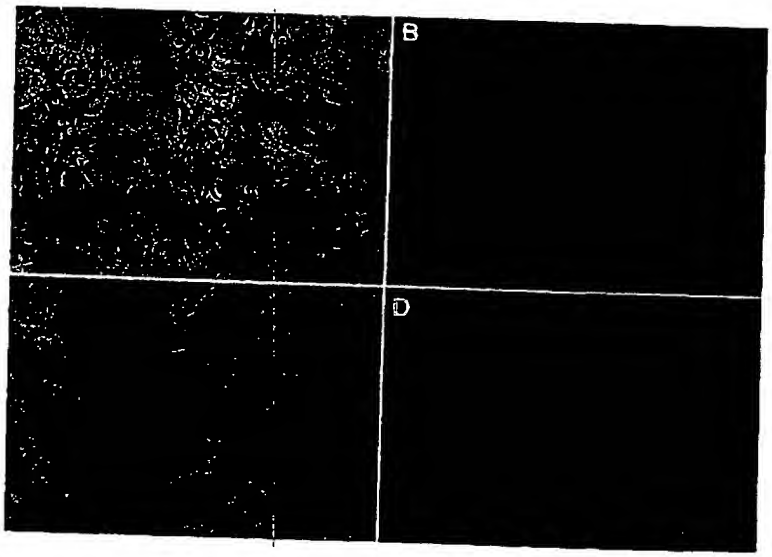

Fig. 2 Expression of recombinant LDL receptor in primary hepatocytes. Primary cultures of hepatocytes were infected with recombinant retroviruses and analysed for LDL receptor expression using an assay based on uptake of fluorescent labelled LDL. Mock infected cells visualized under the phase contrast (*a*) and fluorescent microscope (*b*), and LDL receptor transduced cells visualized under the phase contrast (*c*) and fluorescent microscope (*d*) are presented. Magnification, 100×.

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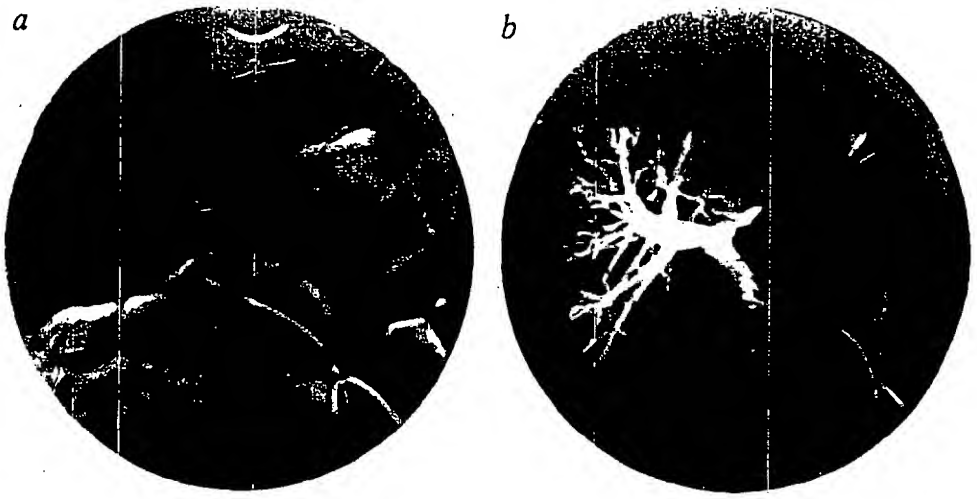

Fig. 3 Portal venograms before and after hepatocyte infusion. *a*, Portal venogram immediately prior to cell reinfusion (postoperative day 3). Note surgical absence of the left lateral segment of the liver, absence of portal vein thrombosis and good position of the catheter. *b*, Portal venogram immediately prior to catheter removal (postoperative day 10). Note patent portal vein without evidence of intraluminal thrombus.

vein thrombosis and/or portal hypertension as a result of introducing a large cell mass into this low pressure venous circulation. Portal pressures measured via the catheter three days before cell infusion (9.8 ± 1.3, mean ± s.d., n=4) were indistinguishable from those measured five days after cell infusion (10.9 ± 2.1 mean ± s.d. n=12) with transient increases (lasting <4 h) of 4 and 8 cm H_2O occurring after the second and third cell infusions, respectively. Repeat portal venography performed at the time of catheter removal seven days after cell infusion revealed a fully patent portal circulation without evidence of intraluminal clot (Fig. 3*b*).

Prolonged improvement in dyslipidemia

Liver tissue was harvested by percutaneous biopsy four months after gene therapy. No histopathology was noted in plastic embedded sections prepared for light and electron microscopy (data not shown). Frozen sections were analysed for the presence of transgene expressing cells by *in situ* hybridization using an RNA probe specific for the recombinant derived LDL receptor transcript. Figure 5 presents an example of an hepatocyte that hybridized to the antisense probe (*c* and *d*); this kind of focal hybridization was not present in serial sections incubated with the sense probe (*a* and *b*) or in sections pretreated with RNase prior to hybridization with the antisense probe (data not shown). Analysis of a limited number of sections revealed hybridization to the antisense probe in 1:1,000 to 1:10,000 cells. It is unlikely that the results obtained from a single small block of liver tissue from FH1 accurately represents the abundance and distribution of transgene expressing cells throughout the liver. Similar experiments performed in three baboons who underwent

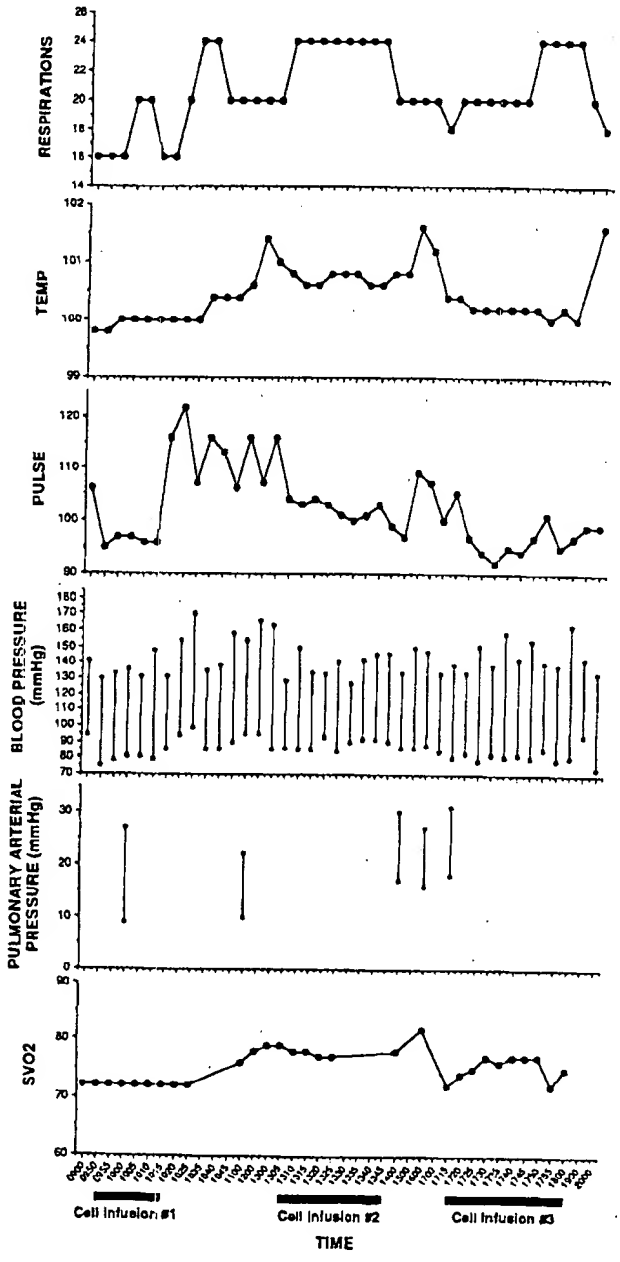

Fig. 4 Clinical response during hepatocyte infusion. During the cell infusions the patient was invasively monitored with a radial arterial line and pulmonary arterial line while in th Intensive care unit. Six clinical parameters (respiration rate, oral temperature, pulse, systemic blood pressure (mm Hg), pulmonary arterial pressure (mm Hg), and oxygen saturation in mixed venous blood (SV02)) are presented. The actual times are indicated along the bottom with the periods during which the cells were infused indicated by the bars. Note that the time coordinates have been expanded during the periods of cell infusion.

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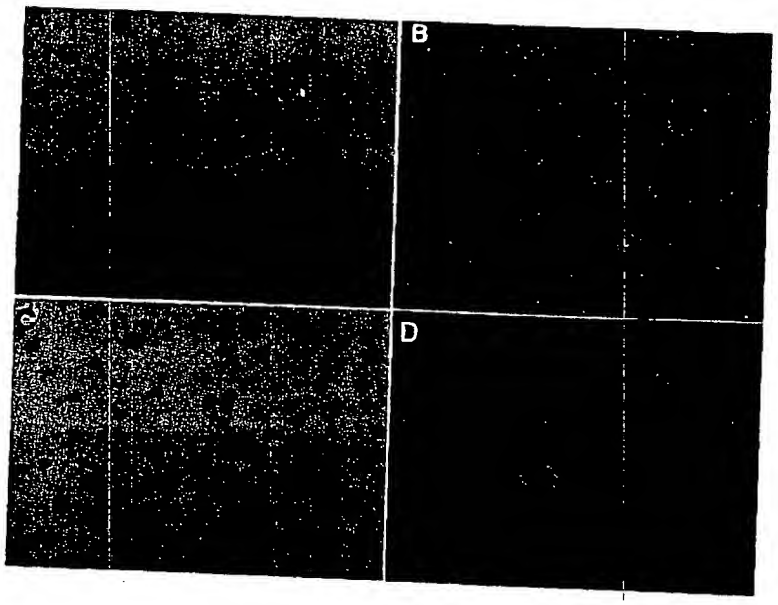

Fig. 5 *In situ* hybridization of liver tissue after gene therapy. Liver tissue (50 mg) was harvested by percutaneous biopsy four months after gene therapy. The majority of the sample was fixed, embedded, sectioned and analysed by light and electron microscopy for evidence of pathology. A small block was analysed for cells expressing recombinant LDL receptor by *in situ* hybridization. Tissue sections were hybridized with the sense probe (*a* and *b*) or antisense probe (*c* and *d*) and visualized by bright field (left panels) and dark field (right panels) microscopy. The clustering of signal seen in panels *c* and *d* indicates a cell that hybridized to the antisense probe. Magnification, 50x.

ex vivo liver-directed gene therapy demonstrated marked regional variation of recombinant LDL receptor expressing cells within large biopsies of liver, illustrating the limitations in quantitatively assessing engraftment from the small quantity of tissue sampled from a percutaneous biopsy (unpublished data).

The effect of gene therapy on the patient's lipid profiles, presented as ΔLDL, ΔHDL and LDL/HDL ratio, is presented in Fig. 6; Table 1 summarizes the lipid data with relevant statistical analyses. The patient has been followed for 18 months after gene therapy in the context of four treatment periods: pre gene therapy — on (period A) and off (period B) lovastatin; and post gene therapy — off (period C) and on (period D) lovastatin. The original protocol was designed to establish baseline lipids when the patient was off all lipid lowering medications and to reinitiate pharmacologic therapy four months after gene therapy. This initial analysis allowed comparisons between periods B, C and D.

Blood samples were coded and submitted to a reference

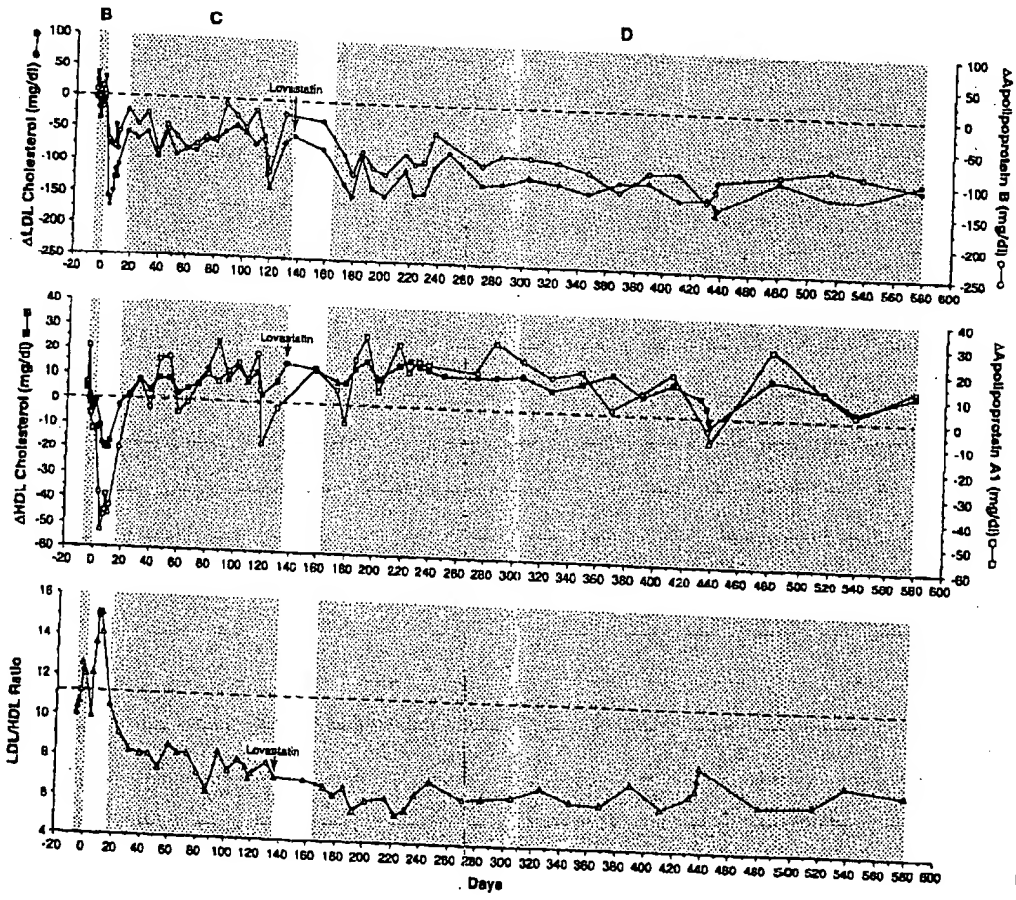

Fig. 6 Lipid profiles. The study was performed using three treatment periods. Period B spans 8 days immediately prior to gene therapy during which 7 lipid profiles were obtained. Period C represents a 131 day interval after gene therapy before she was started on lovastatin during which 19 lipid profiles were obtained. Interpretation of data obtained 8 days following gene therapy was confounded because of additional effects on lipids of the stress of the procedure and decreased nutritional intake; these data were deleted from the analysis of Period C. Period D represents a 15 month interval following Period C during which the patient was treated with lovastatin. Data obtained during a 30 day period after initiation of lovastatin was not included in analysis of Period D to allow for the effect of the drug. Data are presented as ΔLDL and Δapo B (top panel); ΔHDL and Δapo AI (middle panel); and LDL/HDL ratio (bottom panel). Three treatment periods are indicated: period B — pre-gene therapy, off medications; period C — post-gene therapy, off medications; and period D — post-gene therapy, on lovastatin.

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Table 1 Summary of lipid profiles for patient FH1

	Pre-gene therapy		Post-gene therapy		Statistical comparisons (p values)		
	+Lovastatin period A	–Lovastatin period B	–Lovastatin period C	+Lovastatin period D	B–C	C–D	A–D
Cincinnati ref. lab.							
LDL	–	482 ± 19 (7)	404 ± 24 (19)	356 ± 22 (27)	0.0001	0.0001	–
HDL	–	43 ± 3.4 (7)	51.4 ± 5.9 (19)	54 ± 5 (27)	0.0014	0.10	–
LDL/HDL	–	11 ± 1.0 (7)	7.9 ± 0.9 (19)	6.6 ± 0.6 (27)	0.0001	0.0001	–
apoAI	–	115 ± 12 (7)	121 ± 11 (18)	130 ± 9 (24)	0.22	0.005	–
apoB	–	352 ± 23 (7)	299 ± 30 (18)	260 ± 23 (24)	0.0004	0.0002	–
Quebec ref. lab.							
LDL	448 ± 30 (6)	–	–	366 ± 25 (7)	–	–	0.0001
HDL	44.5 ± 4.1 (6)	–	–	47.6 ± 4.1 (7)	–	–	0.16
LDL/HDL	10.2 ± 1.0 (6)	–	–	7.6 ± 0.6 (7)	–	–	0.0001

All samples represent mean ± 1. s.d. with *n*=number of determinations. Statistical analyses performed as described in th text.

laboratory in Cincinnati for analysis. Serum LDL dropped by 180 mg dl^{-1} immediately after cell infusion and regained a new baseline that was 17% lower than pre treatment levels (482±19 before therapy versus 404 ± 24 after therapy, p=0.0001). Coincident with the diminution in LDL was an increase in HDL from 43 ± 3.4 to 51.4 ± 5.9 (p=0.0014) that translated to a decline in LDL/HDL ratio from 11 ± 0.4 to 7.9 ± 0.9 (p=0.0001). The mechanism(s) responsible for increased HDL following gene therapy remain unexplained, however, similar effects have been described in FH homozygotes who underwent orthotopic liver transplantation[2,3]. Initiation of lovastatin four months after gene therapy was associated with further improvements in this patient's dyslipidemia including a reduction in LDL (404 ± 24 to 356 ± 22, p=0.0001), increase in HDL (51.4 ± 5.9 to 54 ± 5, p=0.10), and decline in LDL/HDL ratio (7.9 ± 0.9 to 6.6 ± 0.6, p=0.0001). The changes in LDL and HDL noted in each treatment period were associated with parallel and equally significant changes in apo B and apo AI, respectively (Table 1 and Fig. 6).

An attempt was made to evaluate further the effect of gene therapy using lipid profiles that were obtained in Quebec during period A, spanning a two year interval from Feb. 1990–Dec. 1991; during this time the patient was treated with lovastatin in a single drug regimen at approximately the same dose she has been taking during period D (that is, following gene therapy). Additional samples were obtained during period D and analysed by the same reference laboratory in Quebec that was used to measure the patient's lipids in 1990–91. Direct comparison of the patient's lipids on lovastatin before and after gene therapy revealed a diminution in LDL from 448 ± 30 to 366 ± 25 (p=0.0001), a modest increase in HDL from 44.5 ± 4.1 to 47.6 ± 4.1 (p=0.16), and a decline in LDL/HDL ratio from 10.2 ± 1.0 to 7.6 ± 0.6 (p=0.0001).

Discussion

FH represents a unique model for developing and evaluating the principle of liver-directed gene therapy in humans. This fatal disease is easily evaluated for reconstitution of hepatic gene expression by serial measurements of serum lipids which are considered relevant endpoints for clinical efficacy. Critical to the early development of this clinical model was the availability of an authentic animal model, the WHHL rabbit.

Ex vivo approaches to liver-directed gene therapy emerged as the initial paradigm for treating hepatic metabolic diseases such as FH. In this strategy, stable reconstitution of hepatic gene expression can be achieved by transplanting hepatocytes transduced *ex vivo* with retroviruses. The development of safe and effective *ex vivo* gene therapies to liver presents unique experimental challenges. *Ex vivo* correction of the defect is complicated because the target cell for gene transfer, the hepatocyte, must be isolated from surgically resected tissue and it cannot be maintained and expanded in culture. The ultimate success of this approach depends on the efficient and stable engraftment of the transduced cells and their progeny. The likelihood that this will occur with transduced hepatocytes is difficult to predict because of the paucity of information available regarding stem cells and lineage in the liver, and ultimately must be answered experimentally. Clinical application of this form of gene therapy was further confounded because it does not resemble existing forms of therapy as is the case with bone marrow directed gene therapy, which conceptually is a modification of a commonly used therapy, autologous bone marrow transplantation. However, there should be no immunological barriers associated with *ex vivo* gene therapy other than the problem of an immune response to the therapeutic gene product, a potential concern that is generic to all forms of gene therapy for deficiency states. A variety of animal models, in addition to the WHHL rabbit, have been useful in developing the requisite technology and providing sufficient preclinical studies to justify a human trial[4,9,10].

The outcome of our first clinical experience supports the safety and feasibility of *ex vivo* gene therapy directed to liver. Molecular and metabolic data suggest that the genetically modified hepatocytes have engrafted stably in this patient and continue to express the recombinant gene (after at least 18 months). The level of metabolic correction achieved in this patient was similar to that detected in the WHHL rabbits who received autologous genetically corrected hepatocytes[4]. In this animal model, control experiments performed with mock transfected hepatocytes had no effect on cholesterol except for a transient elevation suggesting that the persistent diminution in lipoproteins observed in FH1 was not an artefact of the surgical

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procedures but due to expression of the recombinant gene. Subsequent to gene therapy, the patient's serum lipids consistently remained at levels significantly lower than those measured by at least two reference laboratories over several years before gene therapy. It is unclear, however, whether the partial correction of hypercholesterolaemia achieved in this patient will translate to improved clinical outcome. It is encouraging that she tolerated gene therapy well without short or long term sequelae and that her coronary artery disease, as documented by serial angiography, has not progressed during the 18 months since the treatment (data not shown).

The response of this patient to lovastatin following gene therapy is interesting given that she failed to respond to this drug on multiple occasions prior to gene therapy. Lovastatin is thought to deplete intracellular cholesterol which leads to up regulation of LDL receptor expression[1], probably at the transcriptional level[11]. The recombinant LDL receptor gene does not contain the transcriptional elements necessary to confer cholesterol mediated regulation suggesting the response to lovastatin is unrelated to the recombinant gene or that its effect is in part mediated by posttranscriptional regulation of LDL receptor. This is consistent with previous studies that indicate the endogenous LDL receptor gene is regulated at both a transcriptional and posttranscriptional manner[12].

One potential concern about gene therapy for diseases caused by loss of gene function is that the protein product of the therapeutic gene will be recognized by the recipient as a neoantigen leading to an immune response against the genetically corrected cells. Several observations suggest this did not occur in FH1. Western blot analysis of the patient's sera failed to detect antibodies to the recombinant human LDL receptor protein (data not shown). Also, there was no clinical or pathological evidence for autoimmune hepatitis following gene therapy. It will be interesting to see if similar results are obtained in FH patients undergoing gene therapy who have mutations that totally ablate LDL receptor protein expression as opposed to the mutation in FH1 that leads to the expression of a dysfunctional protein[7].

Our study demonstrates the feasibility, safety and potential efficacy of *ex vivo* liver-directed gene therapy in humans and supports the initial hypothesis that selective reconstitution of LDL receptor expression in hepatocytes of FH homozygotes should be sufficient for metabolic improvement. This represents the first example of stable correction of a therapeutic endpoint by gene therapy, in contrast to clinical trials that require repeated administration of short-lived target cells such as lymphocytes for treatment of adenosine deaminase deficiency. Translation of this technology to the treatment of other lethal liver metabolic diseases (such as, ornithine transcarbamylase deficiency) should proceed rapidly if the principle of *ex vivo* liver-directed gene therapy is confirmed in a larger number of homozygous FH patients. Ultimately, a more effective and clinically practical approach to liver directed gene therapy, based on *in vivo* gene delivery, must be developed. Gene transfer technologies using recombinant adenoviruses, liposomes and molecular conjugates have shown promising results in animal models[13–15]. Problems with efficiency and stability of recombinant gene expression as well as destructive and/or blocking immune responses to the delivery vehicles or genetically modified cells must be overcome before the potential of *in vivo* approaches can be realized.

Methodology

Surgical procedures. During the procedure and for the first three postoperative days the patient was carefully monitored with a pulmonary arterial catheter and radial arterial line. Following induction of anesthaesia, the left lobe of the liver was exposed by a left subcostal incision which was extended up the midline to the xiphoid. A self-retaining retractor was used to retract the costal margin. The left triangular ligament was divided to the level of the left hepatic vein from lateral to medial. A rubber-shod, non-crushing intestinal bowel clamp was tested for fit just to the left of the falciform ligament. The clamp was applied and using a scalpel, the liver surface was rapidly transected (<90 s) and transferred to the human applications laboratory for cell isolation. Bleeding from the cut hepatic vein was easily controlled with direct pressure until surgical hemostasis was applied. The cut ends of the portal vein and hepatic vein were sutured with 5-0 running non-absorbable monofilament. The open surface of the liver was controlled with 3-0 silk interlocking vertical mattress sutures placed in the liver tissue protruding from the clamp. Once haemostasis was achieved, the clamp was removed and individual bile ducts or blood vessels were ligated with additional 3-0 silk sutures. The inferior mesenteric vein was identified at the paraduodenal fossa. The vein was sharply dissected for a distance of 3 cm and individual branches were ligated with 5-0 silk ligatures. Silk ties (2-0) were placed at either end of the dissected vessel, and the ligature placed at the end of the vein nearest to the colon was tied. A 9.6 Fr. Hickman-type catheter was brought obliquely through the abdominal wall about 3 cm below the lateral aspect of the incision and secured in place with a 3-0 nylon stitch. The catheter was trimmed to the correct length, a bevel was placed at the cut end to facilitate insertion, and the catheter was primed with heparinized saline (100 U ml^{-1}). A venotomy was made with a number 11 scalpel blade. The ideal location for catheter placement is the confluence of the inferior mesenteric vein and the splenic vein, a position that was identified by palpation. The catheter was secured by tying the ligature nearest the portal vein around the inferior mesenteric vein making sure not to occlude the catheter. A 3-0 chromic suture was tied around the inferior mesenteric vein and the outside of the catheter to further protect against premature dislodgement (see Fig. 1). A final inspection of the cut surface of the liver was made and the liver bed was drained with a closed suction drainage catheter to remove any residual bile or serum. The wound was closed in two fascial layers with a running absorbable monofilament suture. The skin was closed with interrupted subcuticular 4-0 chromic sutures and surgical tapes.

Preparation of virus and isolation of hepatocytes. The recombinant retrovirus used in this study has been described[6]. A full length human LDL receptor cDNA is expressed from a chicken β-actin promoter in combination with an enhancer from the immediate early gene of cytomegalovirus. The cell line that produced this virus, called 132-10, was characterized in accordance with recommendations of the RAC and the FDA. Supernatants containing the LDL receptor viruses were cryopreserved and aliquots were analysed for the presence of contaminants and replication competent virus. Certified lots of cryopreserved virus were used in the clinical trial.

Hepatocytes were isolated by collagenase perfusion, plated in culture and infected with retroviruses as described previously[16]. Plates of cells were infected with virus from 132-10 (LDL receptor virus) and analysed for LDL receptor expression using the previously described assay; cells were incubated in RPMI 1640 medium containing lipoprotein deficient serum (10%) and fluorescent labelled LDL (10 μg ml^{-1}) for 4 h[16]. Following completion of this incubation, the medium was removed, and the cells were washed in PBS and visualized under the fluorescent microscope. In preparation for transplantation, hepatocytes were removed from the plates by incubation with trypsin and washed extensively in RPMI 1640. Hepatocytes were harvested in three batches each of which contained cells recovered from one third of the total prep. Each batch was washed and resuspended in normal saline (50 ml) containing 10 U ml^{-1} of heparin in preparation for infusion.

Analyses of biopsied liver tissue. The tissue block for *in situ* analysis

FROM BIOMEDICAL INFORMATION SERVICE (THU) 3. 21'02 14:55/ST. 14:47/NO. 4862641229 P 10

was frozen in OCT, and cryosections (6 μM) were mounted on gelatin poly(L-lysine)-coated sides and fixed with 4% paraformaldehyde in phosphate buffered saline[17]. Sections were hybridized to a ^{35}S labelled RNA probe complementary to retroviral envelope sequences that are uniquely present in the 3' untranslated region of the recombinant derived LDL receptor RNA[4]. Sense probes and RNAse pretreatment with antisense probes were used as controls for hybridization specificity.

Analysis of metabolic parameters. Blood samples were obtained, coded and sent to reference laboratories in Cincinnati and Quebec for determination of lipid profiles. LDL cholesterol, HDL cholesterol, ApoA I and ApoB were measured directly using previously published techniques. Differences in LDL, HDL and LDL/HDL profiles obtained during the four treatment periods were evaluated using random intervention testing and randomized testing methodologies.

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FROM BIOMEDICAL INFORMATION SERVICE (THU) 3. 21' 02 14:56/ST. 14:47/NO. 4862641229 P 11